PATENT
Docket No.: 19603/4230 (CRF D-2238B)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: R. Hutson
Serial No.	:	09/282,239	Art Unit: 1652
Cnfrm. No.	:	8339	
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

SECOND DECLARATION OF MAHENDRA S. RAO, M.D., PH.D. UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, MAHENDRA S. RAO, M.D., Ph.D., pursuant to 37 C.F.R. § 1.132, declare:

1. I received an M.D. (MBBS) degree in Medicine from Bombay University, Bombay India and a Ph.D. degree in Medicine from California Institute of Technology in Pasadena, California.

2. I am a Section Chief for the Stem Cell Unit at the Laboratory of Neuroscience, at NIA (National Institute on Aging), Triad Technology Center, 333 Cassell Drive, Baltimore, MD 21224; an Associate Professor of Neurosciences at Johns Hopkins University School of Medicine, Baltimore, MD 21224; and an Associate Professor at NCBS, Bangalore, India.

3. I am a founder of and shareholder in Q Therapeutics, Inc., 615 Arapeen Drive, Suite 102, Salt Lake City, Utah 84108, which I understand has an exclusive license under the present patent application.

4. I am familiar with the subject matter of the present patent application which I understand is directed to an enriched or purified preparation of human mitotic oligodendrocyte

progenitor cells where the cyclic nucleotide phosphodiesterase 2 promoter (i.e. CNP2) is transcriptionally active in all cells of the enriched or purified preparation.

5. I am a co-inventor of U.S. Patent No. 6,361,996 ("'996 Patent"), which I understand has been used as a basis for rejecting claims in the above application. I present this declaration to demonstrate why the subject matter of the '996 Patent is very different from that of the present patent application.

6. The '996 Patent discloses multipotential neuroepithelial stem cells and lineage-restricted astrocyte/oligodendrocyte precursor cells. The astrocyte/oligodendrocyte precursor cells are derived from neuroepithelial stem cells, are capable of self-renewal, and can differentiate into astrocytes and oligodendrocytes but not neurons. The '996 Patent characterizes these cells as "multipotential intermediate precursor cells restricted to glial lineages" (emphasis added)(column 23, lines 1-5). Similarly, my paper Rao, et. al., "Glial-Restricted Precursors are Derived From Multipotential Neuroepithelial Stem Cells," *Devel. Biol.* 188: 48-63 (1997) clearly demonstrates that such A2B5+/NCAM- cells are capable of generating both astrocytes and oligodendrocytes and do not appear committed to the oligodendrocyte lineage. The '996 Patent's astrocyte/oligodendrocyte precursor cells are in a less differentiated state than the oligodendrocyte progenitor cells of the present patent application and, therefore, are very different from the cells described in this present application.

7. As shown in Figures 1-2 of the '996 Patent, the astrocyte/oligodendrocyte precursor cells 14 and 54, respectively, differentiate directly into two cell types – i.e. of astrocytes and oligodendrocytes. We also know from clonal analysis that there is a homogeneous population of astrocyte/oligodendrocyte precursor cells in which individual cells generate oligodendrocytes and two kinds of astrocytes by the process described in the '996 Patent. It is important to note that multiple pathways to generate post-mitotic, mature oligodendrocytes, have been described. Anderson and colleagues have shown that an oligodendrocyte/motorneuron precursor exists that does not make astrocytes (Zhou et al., "The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification," *Cell* 109:61-73 (2002) (attached hereto as Exhibit 1)). Other investigators have shown distinct sites of origin of oligodendrocytes and astrocytes presumably from separate precursors (Vallstedt et al., "Multiple Dorsoventral Origins of Oligodendrocyte Generation in the Spinal Cord and Hindbrain," *Neuron* 45:55-67 (2005) (attached hereto as Exhibit 2) and Cai et al., "Generation of Oligodendrocyte Precursor Cells from Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling," *Neuron* 45:41-53 (2005) (attached hereto as Exhibit 3)). Yet other investigators have shown that different kinds of oligodendrocyte progenitors exist (Pringle et al., "*Fgfr3* Expression by Astrocytes and Their

Precursors: Evidence that Astrocytes and Oligodendrocytes Originate in Distinct Neuroepithelial Domains," *Development* 130:93-102 (2003) (attached hereto as Exhibit 4)). We are not aware of any evidence that the astrocyte/oligodendrocyte precursor cells of the '996 Patent generated mature oligodendrocytes by way of an intermediate oligodendrocyte-specific precursor. Indeed, Gregori et al., "The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal-Ventral Differences in GRP Cell Function," *J. Neurosci.* 22(1):248-256 (2002) (attached hereto as Exhibit 5) have suggested that the '996 patent describes a glial progenitor that gives rise to a more restricted astrocyte/oligodendrocyte precursor that still directly makes predominantly astrocytes and a small minority of oligodendrocytes. Thus, cells in the '996 Patent's pathway to oligodendrocyte production are bi-potential astrocyte/oligodendrocyte progenitor cells that have strong astrocytic bias. These cell types are very different from the oligodendrocyte-specified progenitor cells of the present application.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 05/10/05

Mahendra S. Rao MD., Ph.D.

Cell, Vol. 109, 61–73, April 5, 2002, Copyright ©2002 by Cell Press

The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification

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Summary

OLIG1 and OLIG2 are basic-helix-loop-helix (bHLH) transcription factors expressed in the pMN domain of the spinal cord, which sequentially generates motoneurons and oligodendrocytes. In *Olig1/2* double-mutant mice, motoneurons are largely eliminated, and oligodendrocyte differentiation is abolished. Lineage tracing data suggest that *Olig1*$^{-/-}$*2*$^{-/-}$ pMN progenitors instead generate V2 interneurons and then astrocytes. This apparent conversion likely reflects independent roles for OLIG1/2 in specifying motoneuron and oligodendrocyte fates. *Olig* genes therefore couple neuronal and glial subtype specification, unlike proneural bHLH factors that control the neuron versus glia decision. Our results suggest that in the spinal cord, *Olig* and proneural genes comprise a combinatorial code for the specification of neurons, astrocytes, and oligodendrocytes, the three fundamental cell types of the central nervous system.

Introduction

The three fundamental cell types of the vertebrate central nervous system (CNS) are neurons, astrocytes, and oligodendrocytes. This basic triad comprises many hundreds or even thousands of distinct neuronal subtypes, in addition to subtypes of astroglia and perhaps of oligodendroglia as well (Raff, 1989; Woodruff et al., 2001). The molecular mechanisms by which these diverse neural cell types are properly generated in space and time are incompletely understood. In recent years, a great deal has been learned about the transcriptional control of the neuron-glial fate decision (Tomita et al., 2000; Nieto et al., 2001; reviewed in Vetter, 2001) and about the control of neuron subtype specification (Briscoe et al., 2000; Jessell, 2000). Rather less is known, however, about the transcriptional control of glial subtype determination.

Two major classes of transcription factors have emerged as determinants of neuron versus glial fate determination and of neuron subtype specification: the basic-helix-loop-helix (bHLH) factors (Vetter, 2001) and homeodomain (HD) factors (Jessell, 2000), respectively. In vertebrates, bHLH factors homologous to the *Drosophila* proneural genes, such as the *Neurogenins (Ngns)* (Gradwohl et al., 1996; Ma et al., 1996; McCormick et al., 1996) and *Mash1* (Johnson et al., 1990), promote neuronal differentiation at the expense of the glial fate (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001b). In the spinal cord, a combinatorial code of HD transcription factors specifies the regional identity of progenitor domains along the dorso-ventral axis (Briscoe et al., 2000; Jessell, 2000). Motoneurons are generated from the pMN domain, while V0, V1, V2, and V3 interneurons are generated from the p0, p1, p2, and p3 domains, respectively (Briscoe et al., 2000; Jessell, 2000). This discontinuous patterning arises from mutually repressive interactions between the HD factors that specify adjacent progenitor domains (Briscoe et al., 2000; Muhr et al., 2001).

Recently, we and others identified a subclass of neural bHLH factors, called *Olig* genes (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). In the mouse, there are two *Olig* genes that are specifically expressed in oligodendrocyte precursors, called *Olig1* and *2* (Lu et al., 2000; Zhou et al., 2000), while in the chick a single gene orthologous to *Olig2* has been identified (Mizuguchi et al., 2001; Zhou et al., 2001). In the spinal cord, oligodendrocyte precursors emerge from a highly restricted domain of the ventral ventricular zone (Miller, 1996; Richardson et al., 2000). This region is precisely demarcated by expression of *Olig1* and *Olig2* (Lu et al., 2000; Zhou et al., 2000). *Olig2* is sufficient to cause ectopic differentiation of oligodendrocytes in the chick spinal cord when misexpressed together with the HD factor Nkx2.2 (Sun et al., 2001a; Zhou et al., 2001), while *Olig1* promotes oligodendrocyte differentiation in rodent cortex (Lu et al., 2001).

Prior to oligodendrogliogenesis, the domain of *Olig2* expression corresponds to the pMN domain, from which motoneurons are generated (Takebayashi et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001). Gain-of-function experiments suggest that OLIG2 plays a determinative role in patterning the pMN domain and also initiates motoneuron differentiation and cell cycle arrest, in part by promoting expression of *Ngn2* (Mizuguchi et al., 2001; Novitch et al., 2001). These data suggest that OLIG2 sequentially controls both motoneuron and oligodendrocyte fate determination. Interestingly, the bHLH factor appears to function in both cases as a transcriptional repressor (Novitch et al., 2001; Zhou et al., 2001).

To rigorously assess the requirement for *Olig* genes in motoneuron and oligodendrocyte differentiation, we have generated double-homozygous mice lacking both *Olig1* and *Olig2*. In *Olig1/2* double mutants, presumptive motoneuron precursors are transformed into V2 interneuron precursors, and oligodendrocytes are lost throughout the brain and spinal cord. Surprisingly, many *Olig2*-expressing oligodendrocyte precursors are transformed into astrocytes. Thus, in the absence of *Olig1/2* function, the sequential production of motoneurons and oligodendrocytes is converted into the sequential production of interneurons and astrocytes. These data suggest that *Olig* genes couple neuronal and glial subtype specification.

Results

Generation of *Olig1* and *Olig2* Double-Mutant Mice

The coexpression of *Olig1* and *Olig2* in vivo (Zhou et al., 2000) raised the possibility that deletion of either of

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these genes alone might be compensated by the function of the other. In order to circumvent this problem, we decided to generate an *Olig1* and *Olig2* double mutant. The mouse *Olig1* and *Olig2* genes are tightly linked on chromosome 16, about 36 kb from each other (data not shown). In order to preserve the *Olig1-Olig2* intergenic region, double-mutant mice were generated through two rounds of homologous recombination in ES cells. The *Olig2* coding region was replaced by a targeting cassette composed of a histone-GFP (hGFP) fusion (Kanda et al., 1998) and PGK-neomycin (Figures 1A and 1B), while the *Olig1* coding region was replaced by a tau-LacZ and PGK-hygromycin cassette (Figures 1D and 1E).

Using a Cre/lox-mediated analytic strategy (see Experimental Procedures), two ES clones were identified in which the *Olig1* and *Olig2* targeted loci lay in *cis* (Figure 1H). Sister cells from these clones, unmodified by Cre recombinase, were injected into blastocysts, and the targeted alleles were transmitted through the germ line (Figures 1C and 1F). Double-heterozygous mice were born at the expected Mendelian frequency and were viable and fertile. However, no live births of homozygous mice were observed, and starting from E18.5, the homozygous embryos appeared smaller than their littermates.

We initially examined the pattern of GFP and LacZ expression in heterozygous *Olig1*$^{+/-}$*Olig2*$^{+/-}$ embryos between E9.5 and E16.5. At E9.5, GFP was strongly expressed in a ventral domain of the spinal cord that corresponds to pMN (Figure 1I), similar to the pattern of endogenous *Olig2* expression (Figure 1J; also see Takebayashi et al., 2000). In contrast, only a few cells were observed to be weakly lacZ positive at this stage (Figure 1N), in agreement with the relatively weak expression of endogenous *Olig1* during the period of neurogenesis (Figure 1O). At E16.0, when *Olig2* expression is restricted to oligodendrocyte precursors (Lu et al., 2000; Zhou et al., 2000), nearly all OLIG2-positive cells were also GFP-positive (Figures 1K–1M, arrows). In addition, GFP colocalized extensively with the *Olig1* knockin marker tau-lacZ (Figures 1P–1R, arrows), consistent with the coexpression of endogenous *Olig1* and *2* in oligodendrocyte precursors (Zhou et al., 2000). Thus, in heterozygous *Olig1*$^{+/-}$*Olig2*$^{+/-}$ mice, the expression of GFP and tau-lacZ faithfully recapitulates the pattern of endogenous *Olig2* and *Olig1* expression in cells of both the motoneuron and oligodendrocyte lineages.

Deletion of *Olig2* and *Olig1* Results in Loss of Motoneurons and a Concomitant Ventral Expansion of V2 Interneurons

We first focused our analysis of *Olig1/2* double-mutant embryos on the generation of several neuronal subtypes derived from the four ventral-most progenitor domains of the spinal cord: En1$^+$ V1 interneurons, Chx10$^+$ V2 interneurons, Isl1/2$^+$ and Hb9$^+$ motoneurons, and Ngn3$^+$ V3 interneurons (Figures 2A–2E). Most Isl1/2$^+$, Hb9$^+$ motoneurons were lost at all axial levels of the homozygous mutant spinal cord at E10.5 (Figures 2H, 2I, and 2K, white bars), while the number of such motoneurons was the same in heterozygote and wild-type (Figures 2C, 2D, and 2K, dark and light gray bars). The loss of motoneurons in the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ spinal cord was not due to cell death, as no increased apoptosis was detected by the TUNEL assay at this stage (Figures 3O and 3T). Although very few presumptive motoneurons (Isl1/2$^+$, Hb9$^+$ cells) were detectable at E10.5 in *Olig1*$^{-/-}$*Olig2*$^{-/-}$ embryos (Figures 2H and 2I, arrowheads), it was possible that a normal number of these neurons might be recovered at later stages through compensatory mechanisms. However, at E13.5, neither somatic (Figures 2L and 2M, arrows) nor visceral (Figures 2L and 2M, arrowheads) motoneurons were detected in the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ spinal cord (Figures 2P and 2Q, arrows and arrowheads, and 2T, white bars). Moreover, no projecting axons were observed in the ventral root (Figures 2O and 2S, arrows), consistent with a lack of both classes of spinal motoneurons.

In contrast to the dramatic loss of motoneurons, the number of Chx10$^+$ cells, which derive from the p2 domain just dorsal to the pMN domain, was increased by about 80% in the double-null mutant spinal cord at E10.5 (Figures 2B versus 2G; 2K, Chx10, white bar). Furthermore, many Chx10$^+$ cells occupied a more ventral position, in territory normally occupied by motoneurons (Figure 2G, yellow arrowhead). The increased number and ventral expansion of Chx10$^+$ V2 interneurons were also apparent at E13.5 (Figures 2N, 2R, and 2T). The number and distribution of En1$^+$ V1 interneurons and Ngn3$^+$ V3 interneurons, by contrast, were largely unaltered in the mutant (Figures 2A, 2E, 2F, 2J, and 2K).

The preceding data suggested that in the absence of *Olig1/2* function, pMN progenitors might give rise to V2 interneurons instead of motoneurons. To confirm this, we used the *Olig2* knockin marker hGFP as a short-term lineage tracer to compare the identities of the neuronal progeny derived from the pMN domain of heterozygous versus homozygous *Olig1/2* double-mutant embryos. In heterozygotes, *Olig2-hGFP*-derived precursors gave rise to Islet1/2$^+$ motoneurons (Figure 3A, yellow cells) but not Chx10$^+$ V2 interneurons (Figure 3B). By contrast, in the homozygotes there were many GFP$^+$, Chx10$^+$ cells present in the marginal zone lateral to the pMN domain of the ventricular zone (Figure 3G, yellow cells). At no time did we detect any Islet1/2$^+$Chx10$^+$ phenotypically hybrid cells (data not shown). In the immediately overlying p2 domain, Chx10$^+$GFP$^-$ V2 interneurons were produced in both heterozygotes and homozygotes (Figures 3B and 3G, white arrowheads). These data strongly suggest that precursor cells from the pMN domain of *Olig1/2* homozygous animals generate V2 interneurons instead of motoneurons.

Irx3 Is Derepressed in pMN in the Absence of *Olig2* and *Olig1* and Respecifies pMN to p2

The loss of motoneurons and concomitant ventral expansion of V2 interneurons in the double-null mutant could reflect a conversion of the pMN domain to a p2 identity. Consistent with this idea, the expression of Irx3, a p2 domain patterning molecule (Briscoe et al., 2000), expanded into the pMN domain in *Olig1*$^{-/-}$*Olig2*$^{-/-}$ double mutants at E10.5 (Figures 3C and 3H, arrows). Pax6 expression, which is high in the p2 domain but lower in the pMN domain (Figures 3D, arrow, and 3E), also

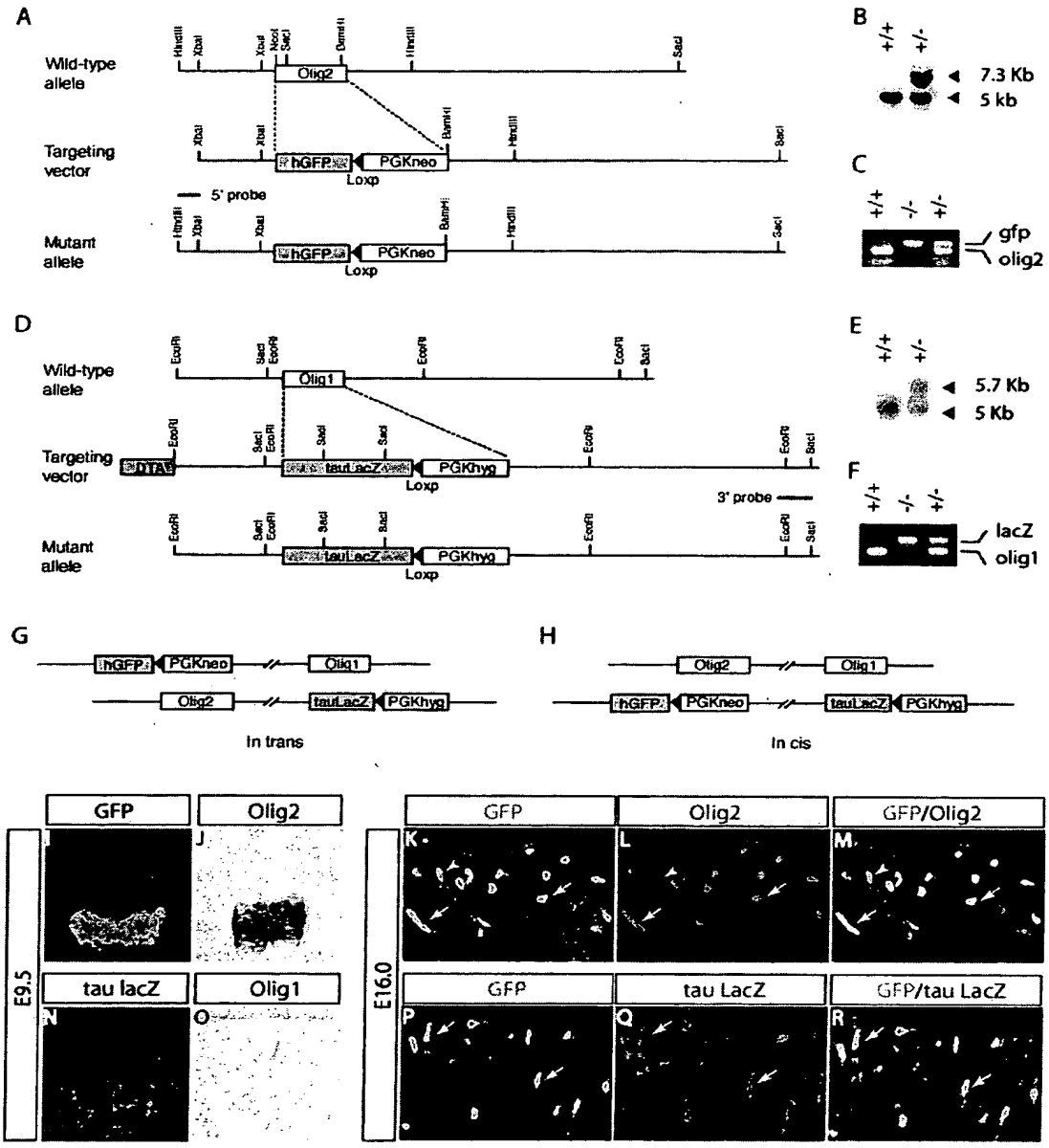

Figure 1. Inactivation of *Olig1* and *Olig2* by Homologous Recombination

(A–C) First round of homologous recombination at the *Olig2* locus.

(A) A *Histone-EGFP/loxp/PGK* neomycin cassette replaced the *Olig2* coding region.

(B) Correct recombination at *Olig2* locus verified by Southern blot analysis of ES clones.

(D–F) Second round of homologous recombination at the *Olig1* locus.

(D) A *tau-LacZ/loxp/PGK* hygromycin cassette replaced the *Olig1* coding region in ES cells in which one *Olig2* locus had been successfully targeted. Abbreviation: DTA, diphtheria toxin A chain.

(E) Correct recombination at the *Olig1* locus verified by Southern blot.

(C and F) Successful germline transmission of the targeted *Olig2* and *Olig1* alleles in *Olig1,2* double-mutant embryos confirmed by genotyping with specific PCR primers.

(G and H) Schematic diagram showing that the two targeting cassettes could lie either in *trans* (G) or in *cis* (H) to each other. A Cre/Loxp analysis was used to identify ES cells in which the two cassettes lie in *cis* (see Experimental Procedures).

(I, J, N, and O) Expression of histone-EGFP and tau-LacZ in the heterozygotes. Thoracic spinal cord sections of E9.5 *Olig1*$^{+/-}$*Olig2*$^{+/-}$ heterozygous embryos were either labeled with anti-GFP antibody (I) or anti-LacZ antibody (N) or were probed by in situ hybridization with cRNAs against Olig2 (J) and Olig1 (O).

(K–M and P–R) Thoracic spinal cord sections from E16.0 *Olig1*$^{+/-}$*Olig2*$^{+/-}$ heterozygous embryos were double labeled with antibodies to GFP and Olig2 (K–M) or to GFP and lacZ (P–R). Extensive colocalization of GFP and Olig2 (K–M) as well as GFP and tau LacZ (P–R) were observed. Arrows indicate double-positive cells. A small number of GFP^{+} cells was found to be Olig2^{-} at this stage (K–M, arrowheads).

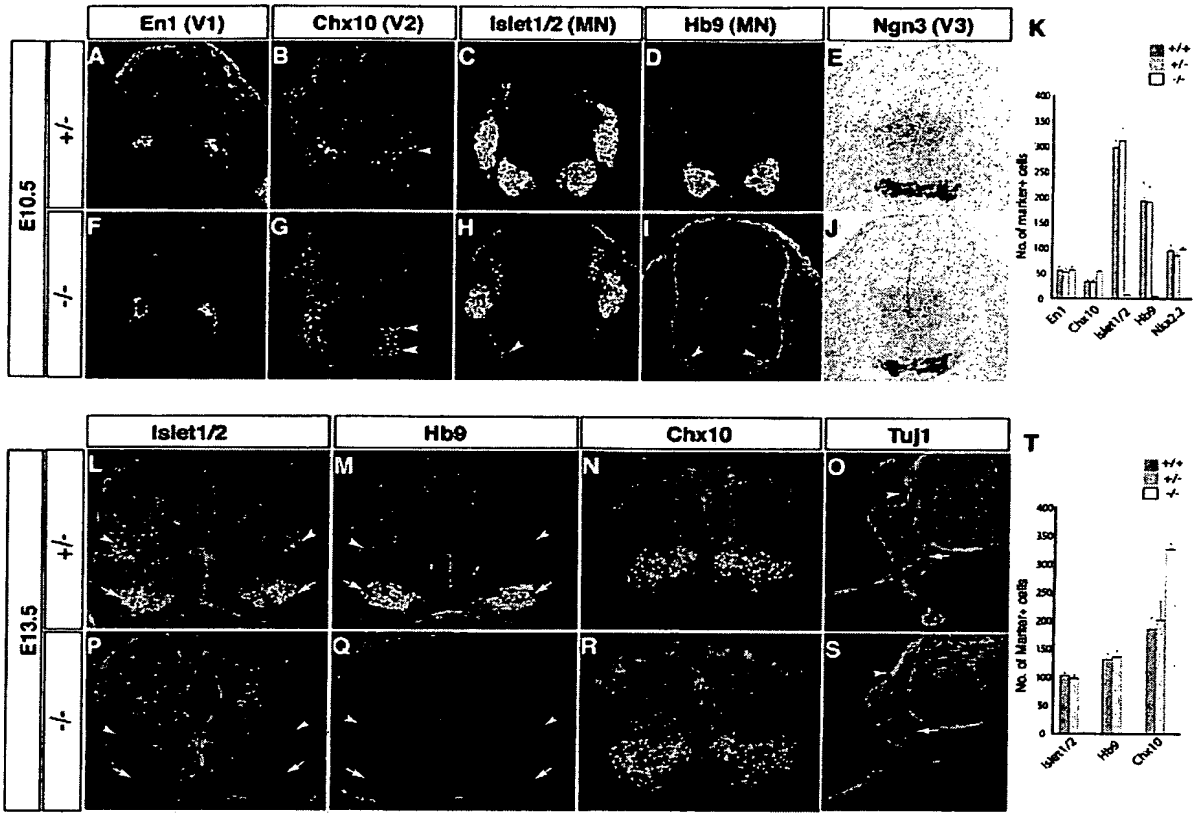

Figure 2. Loss of Isl1/2$^+$Hb9$^+$ Motoneurons and Concomitant Ventral Expansion of Chx10$^+$ V2 Interneurons in the Absence of *Olig1* and *Olig2*

(A–K) Crosssections of E10.5 thoracic spinal cord were stained with antibodies to detect four types of ventral neurons: En1$^+$ V1 interneurons (A and F), Chx10$^+$ V2 interneurons (B and G, arrowheads), Isl1/2$^+$ and Hb9$^+$ motoneurons (C, D, H, and I), and Ngn3$^+$ V3 interneurons (E and J). Note the dramatic reduction of Isl1/2$^+$ and Hb9$^+$ motoneurons in the homozygous spinal cord (C, D, H, and I), while the Chx10$^+$ V2 neurons increased in number and expanded ventrally (B and G, arrowheads). Arrowheads in (H) and (I) indicate the few residual motoneurons formed in the homozygote. White and yellow arrowheads in (B) and (G) indicate Chx10$^+$ V2 interneurons in the p2 and pMN domains, respectively. Quantitative analysis is shown in (K). The number of marker positive cells is presented as mean ± S.D. from nine sections of three embryos. Isl1/2$^+$Hb9$^+$ motoneurons decreased to <5%, and Chx10$^+$ V2 neurons increased ~80% in the double-null mutant compared to heterozygote or wild-type.

(L–T) Motoneuron phenotype at E13.5. Both visceral (L and M, arrowheads) and somatic (L and M, arrows) motoneurons were lost in the homozygotes (P and Q, arrowheads and arrows). In addition, projecting axons were selectively lost in the ventral root of the null mutant (O and S, arrows). Quantitative analysis revealed a 60% increase in the number of Chx10$^+$ cells in the double-null mutant (T; mean ± S.D., six sections from 2–3 animals).

increased in the pMN domain of the null mutant, so that cells in both pMN and p2 were now expressing equally high levels of Pax6 (Figure 3I, arrow). The observed ventral expansion of Irx3 is predicted by the observation that Irx3 and OLIG2 exert crossrepressive activities in gain-of-function assays (Novitch et al., 2001). Surprisingly, however, it did not cause a complete loss of GFP expression from the *Olig2* locus (Figures 3P and 3R), perhaps because the repressive effect of Irx3 is overridden by the higher levels of Shh signaling more ventrally. The expression of several other ventral spinal cord patterning molecules, including Dbx2, Nkx6.1, Nkx6.2, and Nkx2.2 (Briscoe et al., 2000), was unchanged in *Olig1/2* double mutants (data not shown). Taken together, these data suggest that in the absence of *Olig1/2*, pMN cells are converted to a p2 identity (Figures 3E and 3J).

Olig2 and *Olig1* Regulate Neurogenin 2 Expression in pMN

Ectopic expression studies in chick suggested that deletion of *Olig2* and *Olig1* should cause a loss of *Ngn2* expression in the pMN domain (Novitch et al., 2001). Consistent with this prediction, no NGN2-positive cells were detected in the presumptive pMN domain of the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ mutant at E10.0 (Figure 3P), while prominent NGN2 expression was evident in the GFP$^+$ pMN domain of the heterozygous spinal cord (Figure 3K, yellow cells). The lack of apoptotic cells detected by TUNEL labeling in the mutant spinal cord (Figures 3O and 3T) suggests that the loss of NGN2$^+$ cells in the pMN domain does not reflect cell death. We also observed a slight ventral expansion of MASH1 into pMN in *Olig1/2* double mutants (Figures 3L versus 3Q, arrow).

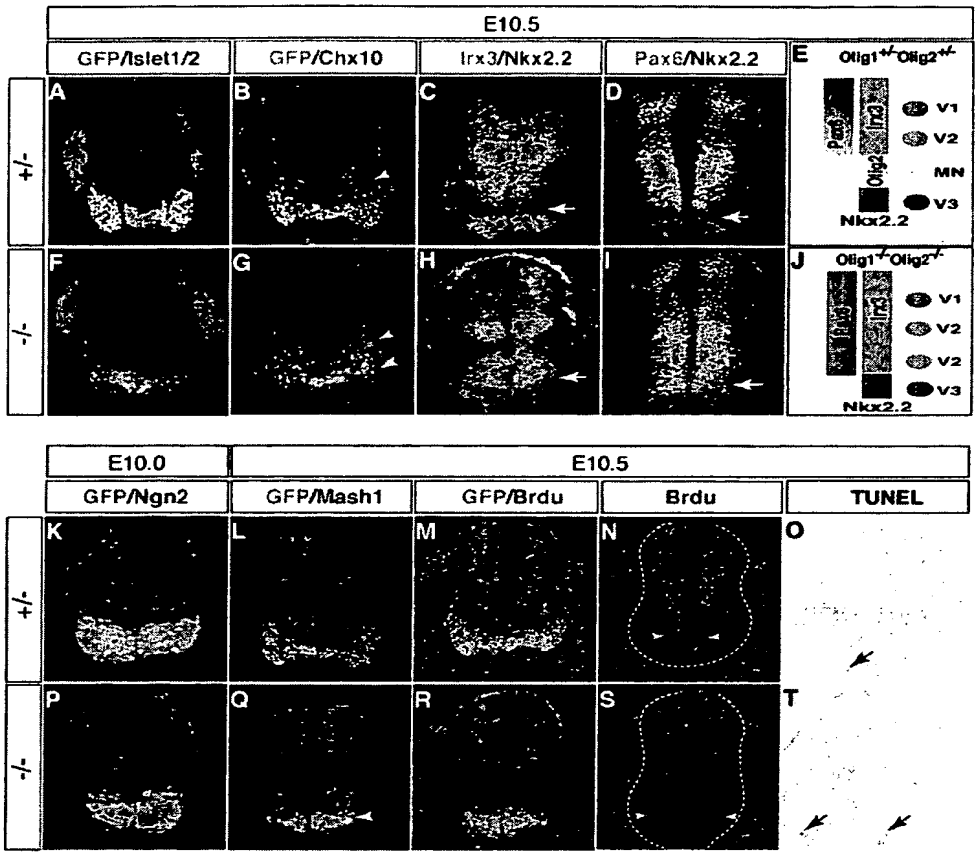

Figure 3. Derepression of *Irx3* and Repression of *Neurogenin2* in the pMN Domain of *Olig1*$^{-/-}$*Olig2*$^{-/-}$ Embryos

(A, B, F, and G) OLIG2-hGFP^{+} precursors in pMN generate Islet1/2^{+} motoneurons in heterozygotes (A, yellow cells) but instead produce Chx10^{+} V2 interneurons in homozygotes (G, yellow arrowhead). White arrowheads in (B) and (G) indicate Chx10^{+}, OLIG2-hGFP^{-} V2 interneurons generated from the p2 domain.

(C and H) Irx3 is derepressed in the pMN of the mutant spinal cord (H, arrow), as is Pax6 (D and I; arrows indicate pMN domain).

(E and J) Summary of the ventral spinal cord patterning defects in *Olig1/2* mutants.

(K, P) Neurogenin 2 (Ngn2) expression is selectively lost in pMN of the mutant at E10.0, while a delayed expansion of MASH1 into this domain is detected at E10.5 (L and Q, arrowhead).

(M, N, R, and S) Many BrdU^{+} cells persist outside the ventricular zone of the pMN in the null mutant (S, arrowheads) compared to heterozygotes (N); the same fields with GFP expression superimposed are shown in (R) and (M), respectively.

(O and T) No significant cell death was observed in the spinal cord at this stage (E10.5). Arrows indicate apoptotic cells outside the spinal cord.

As *Mash1* has recently been shown to be necessary and sufficient for Chx10 expression (Parras et al., 2002), these results may explain how V2 interneurons can differentiate from the mutant pMN despite the absence of NGN2 (Scardigli et al., 2001).

Since NGN2 has been shown to promote cell cycle exit and terminal differentiation (Farah et al., 2000; Novitch et al., 2001), we reasoned that the loss of *Ngn2* expression in pMN might cause delayed cell cycle exit by pMN-derived precursors as they migrated from the ventricular zone. To assess this, we measured BrdU incorporation after a 2 hr pulse in vivo at E10.5. An increased number of BrdU^{+} cells was detected outside the ventricular zone in the GFP^{+} region of the double-null mutant (Figures 3M, 3N, 3R, and 3S, arrowheads). These data suggest that pMN cells lacking OLIG2 fail to exit the cell cycle before migrating into the marginal zone. The presence of ectopic MASH1^{+} cells in pMN is not inconsistent with this observation, as we have recently found that MASH1 promotes cell cycle arrest less efficiently than does NGN2 (Lo et al., 2002).

Failure of Oligodendrocyte Development in *Olig1/2* Double Mutants

We next examined the phenotypic consequences of *Olig2* and *Olig1* deletion on the development of oligodendrocytes. To detect oligodendrocyte precursors, *PDGFRα* and *Sox10* were used as markers (Hall et al., 1996; Zhou et al., 2000), while *MBP* and *PLP/DM20* were used to detect mature oligodendrocytes (Zhou et al., 2001). At no time did we detect expression of any of these markers in the *Olig1/2* double-homozygous mutant at all axial levels of spinal cord examined (Figure 4, −/−, and data not shown) as well as in all brain

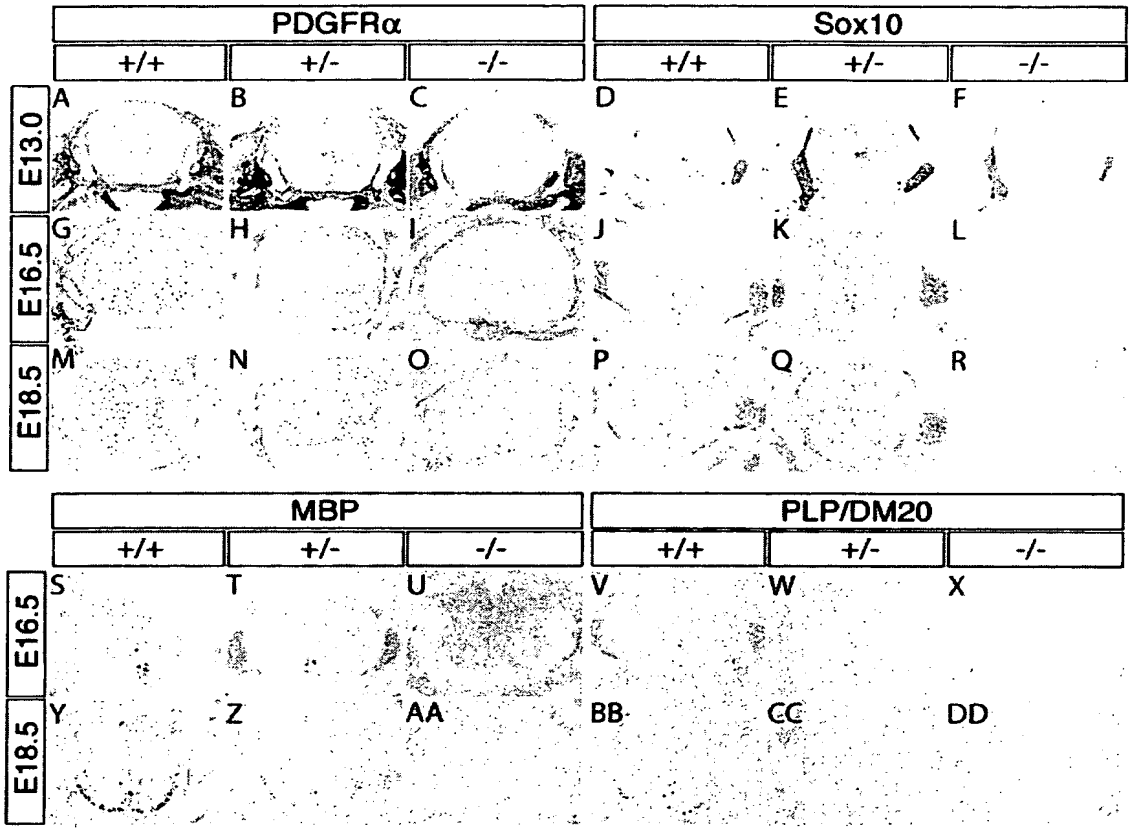

Figure 4. Spinal Cord Oligodendrocytes Fail to Develop in the Absence of *Olig1* and *Olig2*

(A–R) In situ hybridization with the oligodendrocyte precursor markers *PDGFRα* and *Sox10* on crosssections of thoracic spinal cord at indicated stages. Note the total absence of *PDGFRα* and *Sox10* expression in the null mutant spinal cord (C, F, I, L, O, and R).

(S–DD) No *MBP*$^+$ or *PLP/DM20*$^+$ mature oligodendrocytes were detected in null mutant spinal cord (U, X, AA, and DD). In addition, the number of *MBP*$^+$ and *PLP/DM20*$^+$ oligodendrocytes was smaller in the heterozygotes (Z and CC) than in wild-type (Y and BB) at E18.5.

areas examined (Figures 5D–5F and data not shown). In contrast, numerous cells expressing these oligodendrocyte precursor and differentiation markers were present in the wild-type (Figure 4, +/+) and heterozygous (Figure 4, +/−) spinal cord and brain (Figures 5A–5C and data not shown). Thus, there is a total failure of oligodendrocyte formation in the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ double mutant.

Cell counts at E16.5 revealed no decrease in the number of *PDGFRα*$^+$ precursors at thoracic levels of the spinal cord in heterozygotes compared to wild-type (297 ± 17 versus 290 ± 19 cells per 18 μm section, respectively; mean ± S.D., n = 6 sections from three embryos). Consistent with these data, in heterozygotes at E16.5 and P8, *MBP* and *PLP/DM20* expression was normal (Figures 4S, 4T, 4V, and 4W and data not shown). Surprisingly, however, between E18.5 and P0, there was significantly less expression of these mature oligodendrocyte markers in the heterozygotes compared to wild-type (Figures 4Y, 4Z, 4BB, and 4CC and data not shown). These data suggest that a full dosage of *Olig* genes is required for the progression of oligodendrocyte differentiation but not for the initiation of this process.

Olig1 Is Functionally Redundant with *Olig2* in Hindbrain Oligodendrocyte Development

We next examined the phenotype of *Olig1/2* double-knockout embryos in the hindbrain. As in the spinal cord, hindbrain somatic motoneuron differentiation did not occur in *Olig1/2* double mutants, as evidenced by the loss of Isl1/2$^+$, Hb9$^+$ cells and of the XIIth cranial somatic motor nerve (Figures 5G–5I versus 5J–5L, arrows). In contrast, visceral motoneurons, identified by coexpression of Isl1/2 and Phox2b (Dubreuil et al., 2000), were generated (Figures 5J–5L, arrowheads). These results are consistent with the fact that visceral motoneurons in the hindbrain derive from the p3 domain (Briscoe et al., 1999), which does not express either *Olig1* or *Olig2* (data not shown).

In *Olig2*$^{-/-}$ single mutants, oligodendrocytes are spared in the hindbrain while they are lost throughout the spinal cord (Lu et al., 2002 [this issue of *Cell*]). In contrast, we found that neither oligodendrocyte precursors nor mature oligodendrocytes were generated in the hindbrain of *Olig1/2* double mutants (Figures 5D–5F), as was the case in all other brain areas examined (not shown). Taken together, these data suggest that *Olig1*

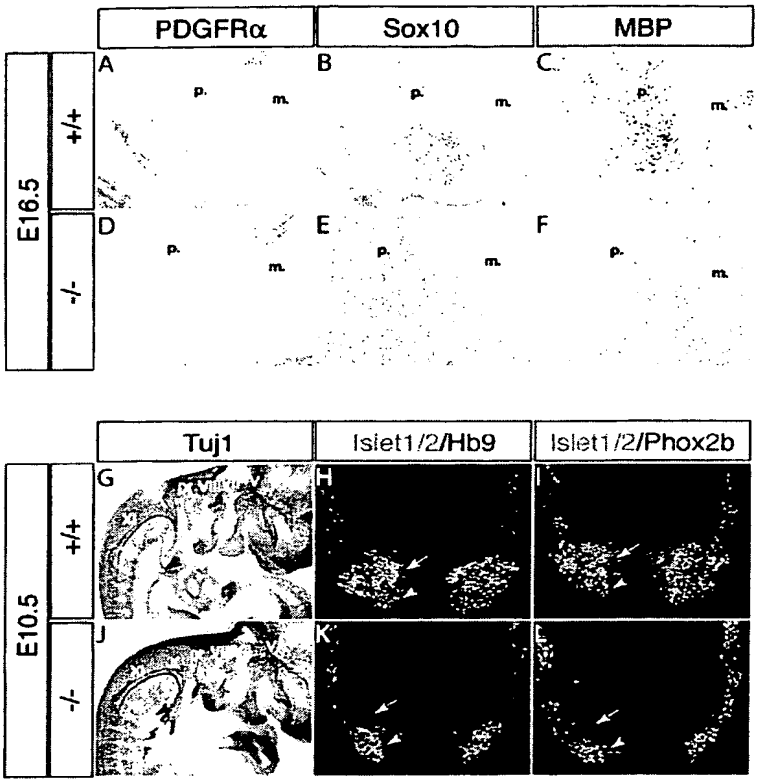

Figure 5. Loss of Hindbrain Oligodendrocytes in the Null Mutant Is Preceded by the Loss of Hindbrain Somatic But Not Visceral Motoneurons

(A–F) In situ hybridization was performed on head saggital sections of E16.5 wild-type or double-null mutant embryos. The pictures were taken from the midbrain-hindbrain region encompassing pons (p.) and medulla (m.). No oligodendrocytes were present in the hindbrain (D–F) as well as other brain areas of the double mutants (data not shown).

(G and J) Whole-mount Tuj1 antibody labeling of E10.5 embryos. The XIIth cranial motor nerve (hypoglossal, arrowhead) was missing from the homozygote.

(H–L) In the caudal hindbrain of the null mutant, Isl1/2$^+$ and Hb9$^+$ somatic motoneurons were lost (K and L, arrows), while Isl1/2$^+$ and Hb9$^-$ visceral motoneurons were still present (K and L, arrowheads).

can compensate for the lack of *Olig2* in oligodendrocyte (but not somatic motoneuron) generation in the hindbrain but not in other regions of the CNS.

Oligodendrocyte Precursors Are Transformed into Astrocytes in the Absence of *Olig2* and *Olig1*

The absence of oligodendrocyte precursors in *Olig1/2* double-mutant embryos could reflect a failure of specification, their death, or their respecification into other cell types. To distinguish between these possibilities, we first tested whether there was increased cell death in the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ spinal cord from E12 to E14, a period when oligodendrocyte precursors are specified in the ventricular zone. TUNEL labeling detected no increase in apoptotic cells in either the ventricular zone or elsewhere in the spinal cord during this interval (data not shown). Next, we used the knockin marker hGFP as a short-term lineage tracer to compare the fate of *Olig2*-expressing progenitors in the presence or absence of *Olig1/2* function.

In heterozygous embryos at E13.5, individual GFP$^+$ precursors could be seen migrating away from the focus of *Olig2* expression in the ventricular zone (Figure 6A, arrow and white arrowheads). By E16.5, only a few GFP$^+$ cells remained at this focus (Figure 6B, arrow), and most had migrated into the gray matter. By E18.5, GFP$^+$ oligodendrocyte precursors were evenly distributed throughout the spinal cord, and ventricular expression was no longer detected (Figure 6C). The pattern of migration of GFP$^+$ cells in the heterozygous spinal cord closely resembles that revealed by antibody staining for endogenous OLIG2 protein (Figures 1I–1K, arrows and data not shown).

In homozygous mutant embryos, the distribution of GFP$^+$ cells was different in several respects. First, although many GFP$^+$ cells were present in the ventricular zone at E13.5 (Figure 6D, arrow), there was little migration into the gray matter. Second, the ventricular focus of GFP expression appeared larger in homozygous than in heterozygous embryos (Figures 6A versus 6D, arrows). Cell counts indicated a similar number of GFP-expressing cells in the null mutant versus heterozygous spinal cord at this stage (Figure 6G, E13.5), suggesting that *Olig2*-expressing cells may have been generated in correct numbers in the homozygote but somehow failed to migrate on schedule. At E16.5 and E18.5, however, there was a reduction in the number of GFP$^+$ cells in the double mutant (Figure 6G, white bars). This difference likely reflects reduced proliferation rather than death, as TUNEL labeling revealed no differences between the double mutant and heterozygote at these stages (data not shown).

By E16.5, although ventricular expression of GFP in homozygotes persisted (Figure 6E, arrow), GFP$^+$ cells could be seen migrating into the gray matter (Figure 6E, arrowheads). However, these cells took a more ventral trajectory than in heterozygotes. At E18.5, many GFP$^+$ cells could be detected at the pial surface of the ventral white matter in homozygotes (Figure 6F, open arrowheads), a location not occupied by GFP$^+$ cells in hetero-

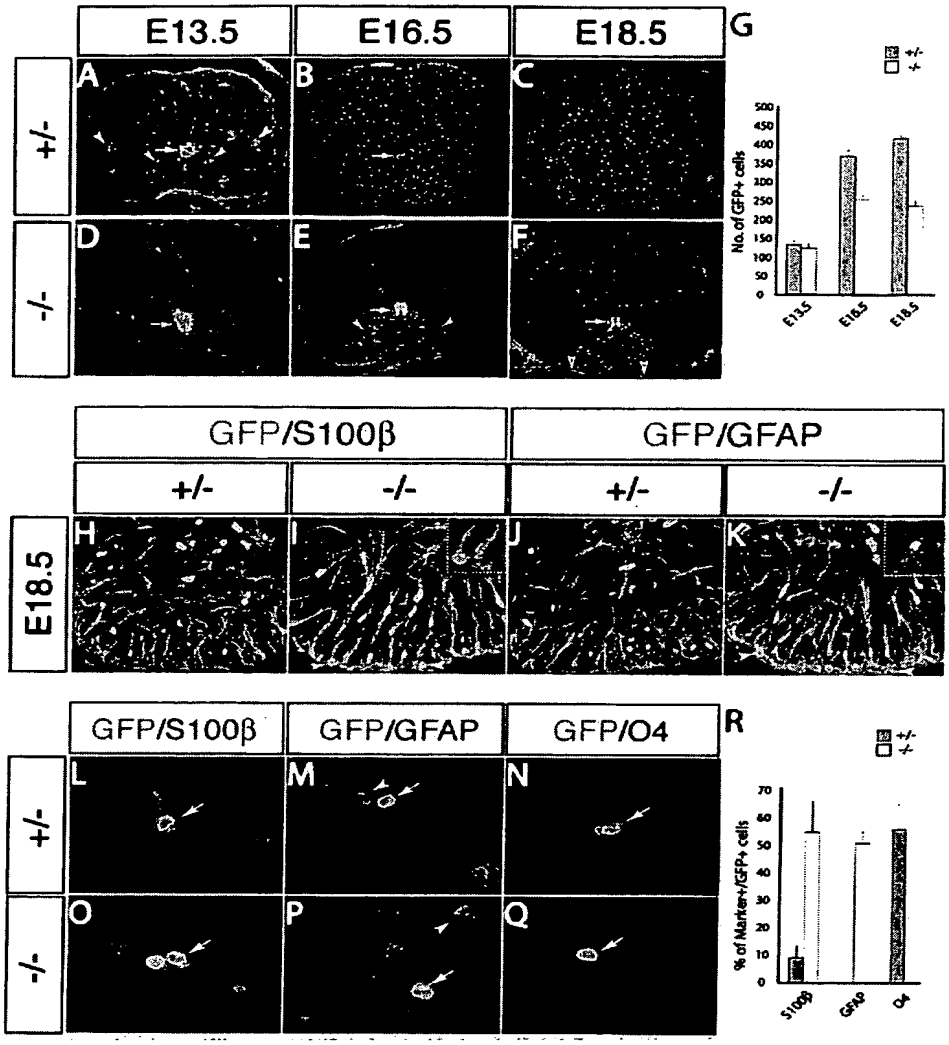

Figure 6. Oligodendrocytes Are Transformed into Astrocytes in the Absence of *Olig1* and *Olig2*

(A–G) Crosssections of heterozygous and homozygous thoracic spinal cord at indicated stages were labeled with an anti-GFP antibody. Arrows indicate ventricular expression of GFP. Persistence of GFP was apparent in both somatic (A, red arrowheads) and visceral (A, yellow arrowheads) motoneurons in the heterozygotes at E13.5, a time when the migration of GFP$^+$ oligodendrocyte precursors just started (A, white arrowheads). Open arrows in (F) indicate GFP$^+$ cells located within pial surface. Quantitative analysis (G; mean ± S.D., eight sections from two embryos) revealed a difference in the number of GFP$^+$ cells in the null mutants versus heterozygotes at E16.5 and E18.5, but not E13.5. (H–K) Many GFP$^+$ nuclei are associated closely with S100β$^+$or GFAP$^+$ fibers in the ventral spinal cord of homozygotes (I and K) but not of heterozygotes (H and J) at E18.5. Arrows point to GFP$^+$ cells in the pial surface of the null mutant spinal cord. Inserts in (I) and (K) show double-labeled cells at higher magnification.

(L–R) Staining of acutely dissociated spinal cord cells at E18.5 from either heterozygotes (L–N) or homozygotes (O–Q). Arrows point to GFP$^+$ cells. Arrowheads in (M) and (P) mark GFP$^-$GFAP$^+$ cells. Quantitative analysis (R) was performed by counting all GFP$^+$ cells from six different preps derived from different animals. Each prep contained ~5000 cells.

zygotes (Figure 6C). This observation suggested that the GFP$^+$ cells might have been transformed into astrocytes. To address this possibility, crosssections of E18.5 heterozygous or homozygous spinal cord were double labeled with antibodies to GFP and the astroglial markers GFAP or S100β. This analysis revealed that many GFP$^+$ cells in the null mutant spinal cord coexpressed GFAP or S100β (Figures 6I and 6K, arrowheads and insets). In contrast, no colocalization of GFP with either of these markers was observed in the heterozygous spinal cord (Figures 6H and 6J), consistent with previous reports that *Olig2* is not expressed in cells of the astroglial lineage in wild-type embryos (Lu et al., 2000; Zhou et al., 2000).

The filamentous staining pattern of GFAP and S100β precluded an accurate quantification of the percentage of GFP$^+$ cells that were GFAP$^+$ or S100β$^+$ in vivo. To circumvent this problem, we performed double-labeling on acutely dissociated spinal cord cells from E18.5 heterozygous and homozygous embryos. Over 50% of GFP$^+$ cells in homozygous spinal cord coexpressed GFAP (Figures 6P, arrow, and 6R, GFAP, white bar). In sharp contrast, none of the GFP$^+$ cells in the heterozygous spinal cord was found to be GFAP$^+$ (Figures 6M, arrow versus arrowhead, and 6R, gray bars). Similarly, 44% to 66% of GFP$^+$ cells were S100β$^+$ in the homozygote (Figures 6O, arrow, and 6R, white bar), whereas less than 10% of GFP$^+$ cells in the heterozygote were S100β$^+$ (Figures 6L, arrow, and 6R, gray bar). Conversely, the oligodendrocyte cell-surface marker O4 decorated over 55% of GFP$^+$ cells in the heterozygous spinal cord (Figures 6N, arrow, and 6R, gray bar) but none in the homozygote (Figures 6Q, arrow, and 6R, white bar). The reciprocal change in the percentage of GFP$^+$ cells expressing O4 versus GFAP or S100β in heterozygous versus double homozygous embryos (Figure 6R) strongly suggests that in the absence of *Olig1/2* function, the *Olig2*-expressing cell population produces astrocytes rather than oligodendrocytes. Consistent with this conclusion, in cultures of neural progenitors from *Olig1/2* homozygous embryonic spinal cord, many Olig2-hGFP-expressing cells differentiated to astrocytes but none differentiated to oligodendrocytes, whereas in cultures from heterozygotes, many GFP$^+$ oligodendrocytes developed (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/109/1/61/DC1).

Discussion

The bHLH transcription factors OLIG1 and OLIG2 are sequentially expressed in motoneuron progenitors (Takebayashi et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001) and oligodendrocyte precursors (Lu et al., 2000; Zhou et al., 2000). Here we show that in the absence of *Olig1* and *2* function, motoneurons are converted to V2 interneurons in the spinal cord, while oligodendrocytes fail to differentiate throughout the nervous system. Our results suggest that oligodendrocyte precursors are not simply eliminated, but instead differentiate to astrocytes. These observations are consistent with the idea that in *Olig1/2* double mutants, *Olig2*-expressing progenitors sequentially generate interneurons and astrocytes rather than motoneurons and oligodendrocytes. In this way, *Olig* genes link the specification of a particular neuronal subtype to that of a specific glial subtype, independent of the decision between neuronal versus glial fates.

Olig2 Is Required for Both the Regional Identity and Differentiation of Motoneuron Precursors

Misexpression studies in the chick have suggested that OLIG2 plays two roles in motoneuron fate determination: it specifies the regional identity of the pMN domain via repression of *Irx3*, and it promotes motoneuron progenitor cell cycle exit and differentiation, in part via local derepression of *Ngn2* (Mizuguchi et al., 2001; Novitch et al., 2001). The loss-of-function data presented in this and the companion paper (Lu et al., 2002 [this issue of *Cell*]) strengthen this view. Combined deletion of *Olig2* and *Olig1* causes a derepression of *Irx3* in pMN and a loss of *Ngn2* expression in this domain. The selective loss of *Ngn2* expression in pMN is consistent with the idea that this bHLH factor is controlled by distinct *trans*-acting factors in different progenitor domains (Scardigli et al., 2001). The motoneuron deficit in the *Olig1/2* double knockout is similar to that seen in embryos lacking *Nkx6.1/6.2* (Vallstedt et al., 2001), a homeodomain patterning molecule (Briscoe et al., 2000) that is required for *Olig2* expression (Novitch et al., 2001). The fact that expression of *Nkx6.1* and *6.2* is unperturbed in *Olig1/2* knockouts is consistent with the idea that *Olig* genes function downstream of *Nkx6.1/6.2* in motoneuron generation (Novitch et al., 2001).

Olig Genes Function Cell Autonomously in Oligodendrocyte Fate Specification

The complete failure of oligodendrocyte formation in *Olig1/2* double mutants suggests that all oligodendrocytes require *Olig* genes. Consistent with this, oligodendrocytes are not generated in neurosphere cultures derived from *Olig1*$^{-/-}$*2*$^{-/-}$ spinal cord (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/109/1/61/DC1). The fact that *Olig1/2* are coexpressed in oligodendrocyte precursors (this study; Lu et al., 2000; Zhou et al., 2000) suggests that this defect likely reflects a cell-autonomous requirement for these genes. An alternative explanation, however, is that this phenotype is a non-cell-autonomous consequence of the earlier loss of motoneurons, which have been hypothesized to send a feedback signal to the ventricular zone to regulate the subsequent production of oligodendrocytes (Hardy, 1997; discussed in Richardson et al., 2000).

We think this hypothesis is unlikely, however, because in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ double mutants, neuronal differentiation in the ventral spinal cord is largely eliminated (Scardigli et al., 2001), but oligodendrocyte precursor formation is unaffected (our unpublished observations). Similarly, in *Isl1*$^{-/-}$ mice which lack motoneurons (Pfaff et al., 1996), oligodendrocyte differentiation is also unaffected (Sun et al., 1998). Finally, oligodendrocytes develop normally in the hindbrain of *Olig2* single mutants (Lu et al., 2002 [this issue of *Cell*]), which lack somatic motoneurons. Our observation that in *Olig1/2* double mutants, hindbrain oligodendrocytes are completely lost further indicates that the sparing of hindbrain oligodendrocytes in *Olig2*$^{-/-}$ embryos is not due to compensation of a somatic motoneuron-derived signal by visceral motoneurons, which are spared in both *Olig2*$^{-/-}$ and *Olig1*$^{-/-}$*2*$^{-/-}$ mutants.

Role of *Olig* Genes in Motoneuron and Oligodendrocyte Fate Specification

The inference that *Olig* genes function cell autonomously in oligodendrocyte development leaves open the question of when that function is required. Our data indicate that both motoneurons and oligodendrocytes are normally generated from pMN (but not from p2) and support the idea (Richardson et al., 1997, 2000) that these neurons and glia share a common precursor. Consequently, the homeotic-like transformation of such pre-

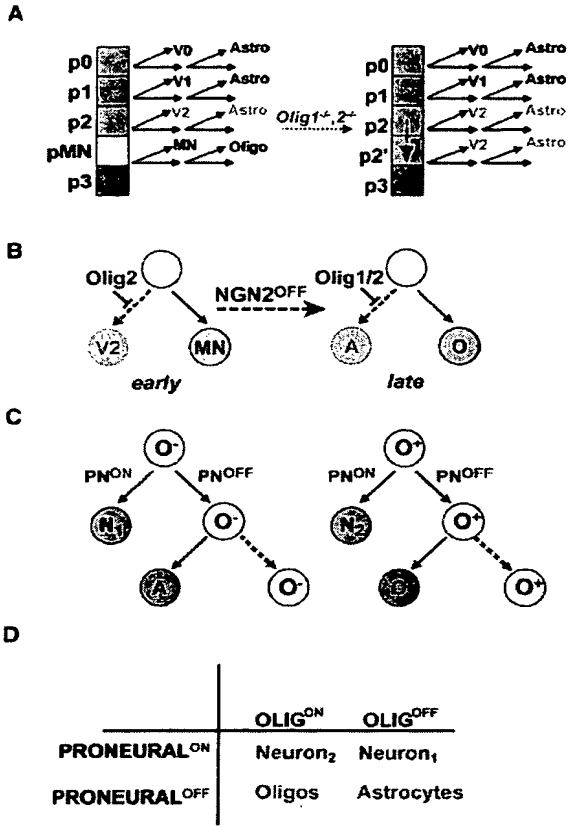

Figure 7. *Olig* Genes Control the Subtype Identities of Both Neurons and Glia Derived from a Common Progenitor Domain

(A) Summary of neuronal and glial phenotypes in *Olig1*$^{-/-}$*Olig2*$^{-/-}$ mutants.

(B) *Olig* genes may act sequentially in motoneuron (MN) and oligodendrocyte (O) development. Abbreviation: A, astrocyte.

(C) Two putative types of spinal cord progenitor cells. O$^-$ progenitors do not express *Olig* genes and generate certain types of neurons (N_1) and later astrocytes (A). Abbreviation: PN, proneural. O$^+$ progenitors first generate other types of neurons (N_2) and then oligodendrocytes (O, blue cell).

(D) A simple combinatorial code composed of proneural and *Olig* genes can determine whether CNS progenitors produce neurons, oligodendrocytes, or astrocytes. Neuron$_1$ and Neuron$_2$ denote two different neuronal subtypes.

cursors from a pMN to a p2 identity in *Olig1/2* homozygous embryos could result in the elimination of both cell types (Figure 7A). In that case, the wild-type function of *Olig* genes in specifying both the motoneuron and oligodendrocyte fates might simply be repression of *Irx3* in pMN. Alternatively, the mutant phenotype could reflect an independent and sequential requirement for *Olig* genes in both early patterning of the pMN domain and in oligodendrocyte fate determination (Figure 7B).

Consistent with the idea of independent functions, the hindbrain phenotype of *Olig2*$^{-/-}$ single mutants demonstrates that *Olig* gene mutations can cause deficiencies in somatic motoneuron generation without necessarily affecting oligodendrocyte development (Lu et al., 2002 [this issue of *Cell*]). Furthermore, while misexpression of *Olig2* in chick is sufficient to cause ectopic repression of *Irx3* and motoneuron induction in some regions of the spinal cord (Novitch et al., 2001), it does not induce ectopic oligodendrocyte differentiation (Zhou et al., 2001). Thus, while repression of *Irx3* by *Olig* genes may be necessary for oligodendrocyte fate determination, it may not be sufficient. Formal resolution of this issue will require selective rescue of the early pMN phase of *Olig2* expression in *Olig1/2* double knockouts to determine whether both motoneurons and oligodendrocytes are recovered.

OLIG1 and 2 Control an Oligodendrocyte versus Astrocyte Fate Choice

Our lineage-tracing data suggest that many *Olig2-hGFP*-expressing precursors generate astrocytes instead of oligodendrocytes upon deletion of *Olig1* and *Olig2*. If so, it would imply that spinal cord oligodendrocyte precursors have the potential to generate astrocytes, but that this fate is normally repressed by *Olig* genes (Figure 7B, right). Astrocytes are thought to arise from multiple levels along the dorso-ventral axis of the spinal cord (Pringle et al., 1998). Thus, it is a reasonable assumption that the p2 domain normally generates astrocytes after it generates V2 interneurons (Figure 7A, left). If so, then the trans-fating of *Olig2*-expressing progenitors to astrocytes in *Olig1/2* double mutants could reflect a conversion of progenitors that sequentially generate motoneurons and oligodendrocytes to ones that produce first V2 interneurons and then astrocytes (Figure 7A, right, p2').

If deletion of *Olig* genes causes oligodendrocyte precursors to generate astrocytes, do such precursors normally generate astrocytes following downregulation of *Olig* gene expression in the ventricular zone? We found no evidence for persistence of the OLIG2-hGFP lineage marker into astrocytes in *Olig1/2* heterozygotes. Similarly, using a permanent lineage tracer, Lu et al. (2002 [this issue of *Cell*]) found no astrocytes among the progeny of *Olig1*-expressing cells. These data suggest that *Olig*-expressing progenitors normally produce motoneurons and oligodendrocytes but not astrocytes in vivo (Figure 7C, O$^+$). This idea may seem inconsistent with the demonstration that single CNS progenitors can generate neurons, astrocytes, and oligodendrocytes in culture (reviewed in Gage, 2000; Anderson, 2001). However, despite extensive retroviral lineage tracing studies, there is no clear evidence for tripotential neuron/astrocyte/oligodendrocyte progenitors in vivo (Luskin et al., 1988; Leber et al., 1990). The tripotential CNS stem cells characterized in vitro may thus represent a more primitive progenitor than has been identified in vivo. Alternatively, the cell culture environment may reveal a combination of developmental potentials that are not actually used by any single progenitor in vivo.

The Genetic Logic of Neural Cell Fate Determination

bHLH proneural genes such as the *Ngns* control a neuron versus glial fate switch (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001b; reviewed in Vetter, 2001). In

the case of motoneurons and oligodendrocytes, *Ngn1/2* is likely to be the proneural gene that controls this switch (this study; Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). These data, taken together with our previous results (Zhou et al., 2001), suggest that once expression of *Ngns* has been extinguished in pMN, *Olig* genes determine whether the remaining progenitors will produce oligodendrocytes or astrocytes (Figure 7B).

These results suggest a simple combinatorial code whereby different combinations of the *Olig* and proneural genes can specify either neuronal, oligodendroglial, or astroglial fates (Figure 7D). According to this genetic logic, the astroglial fate would represent a final "ground state," in which neither proneural nor *Olig* genes are expressed. This fate may, however, require active repression by *Hes* genes, which repress proneural gene expression (Ishibashi et al., 1995; Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001; reviewed in Fisher and Caudy, 1998).

Combinatorial codes of Lim HD and Ets domain transcription factors have been shown to control different aspects of motoneuron subtype identity (Tsuchida et al., 1994; Lin et al., 1998; Sharma et al., 1998; Kania et al., 2000). By contrast, bHLH factors have until now been viewed as primarily acting in linear cascades to produce a single cell type, such as muscle or neuron (Weintraub, 1993; Lee, 1997). The results presented here suggest that in the nervous system, bHLH factors can also function in a combinatorial code that determines the three fundamental cell types of the CNS. This code may provide a foundation upon which higher-order aspects of neuronal and glial subtype identity can be built by superimposing combinatorial codes composed of other families of transcription factors. The linking of these multiple coding systems may then be achieved by crossregulatory, and perhaps physical, interactions between the molecules that comprise them.

Experimental Procedures

Generation of Olig1 and Olig2 Double-Mutant Mice

All mouse genomic clones were derived from a 129SVJ genomic library (Stratagene). Both the mouse *Olig1* and *Olig2* sequences are encoded by a single exon. The *Olig2* targeting vector was constructed by inserting a *Histone-GFP fusion/loxp/PGKneo* cassette between a 2 kb 5′ arm and a 3.8 kb 3′ arm. For the *Olig1* targeting vector, a *tau LacZ/loxp/PGKhyg* cassette was cloned between the 1.9 kb 5′ arm and the 3.2 kb 3′ arm.

Two rounds of electroporation and selection were conducted, first with the *Olig2* targeting vector, and then with the *Olig1* targeting vector. Correctly recombined clones at both the *Olig2* and *Olig1* loci were subjected to Cre/Loxp analysis to determine whether the two recombined alleles reside on the same chromosome (protocol available upon request). The frequency of recombination for both the *Olig2* and *Olig1* loci was ~1:300. Clones in which the two mutant alleles are located in *cis* were injected into C57BL/6J blastocysts to generate germline chimeric founders. Mutant mice were genotyped with PCR primers specific to *Olig1*, *Olig2*, *GFP*, *lacZ*, *Neomycin*, and *Hygromycin* genes. No segregation of the two mutant alleles has been observed in all embryos genotyped so far. All embryos analyzed in this study were derived from heterozygous 129sv × C57BL/6J intercrosses.

In Situ Hybridization

Nonradioactive in situ hybridization was performed as previously described (Zhou et al., 2000). The following mouse gene probes were used: *Olig1*, *Olig2*, *Olig3*, *Sox10* (a gift of Dr. Kirsten Kuhlbrodt), *PDGFR*, *MBP*, *PLP/DM20*, *Ngn1*, and *Ngn3*. Probes for *Nkx6.1*, *Nkx6.2*, and *Dbx2* were the kind gift of Dr. Thomas Jessell.

Immunohistochemistry

Mouse embryos were fixed by immersion in 4% paraformaldehyde from 1 hr to overnight at 4°C depending on the age. The following primary antibodies were used: rabbit anti-Olig2 (1:2000, gift of Dr. Takebayashi Hirohide), rabbit anti-Nkx2.2 (1:1000, gift of Dr. Thomas Jessell), mAb anti-Neurogenin2 (1:100, Liching Lo), rabbit anti-Chx10 (1:4000, gift of Dr. Thomas Jessell), guinea pig anti-Irx3 (1:1000, gift of Dr. Thomas Jessell), rabbit anti-Hb9 (1:2000, gift of Dr. Samuel Pfaff), rabbit anti-GFP and Alexa-488 conjugated rabbit anti-GFP (1:1000, Molecular Probes), rabbit anti β-gal (1:1000, 5′-3′), rabbit anti-GFAP (1:1000, DAKO), mAb anti-S100 β (1:1000, Sigma), and rabbit anti-Phox2b (1:700, gift of Dr. Jean-Francois Brunet). mAbs against Lim3, MNR2/Hb9, Engrailed-1, Isl1/2, Hb9, Lim3, Nkx2.2, and Pax6 were obtained from Developmental Studies Hybridoma Bank (DSHB). Whole-mount antibody staining of mouse embryos was performed as described previously (Ma et al., 1996).

BrdU Labeling and TUNEL Assay

BrdU labeling of mouse embryos was conducted by intraperitoneal injection of BrdU (Sigma, 65 mg/g body weight) 2 hr before sacrifice. A rat anti-BrdU antibody (Accurate) was used to detect BrdU. TUNEL assays were performed with a kit from Roche according to the manufacturer's instructions.

Acknowledgments

We thank Drs. Richard Lu, Charles Stiles, and David Rowitch for communicating unpublished results and Tom Jessell for advice, reagents, helpful discussions, and critical comments on the manuscript. We are also grateful to Shirley Peace and Bruce Kennedy of the Caltech Transgenic Facility, Joana Yamada for technical assistance, and Gaby Mosconi for laboratory management. This work was supported by National Institutes of Health Grant RO1-NS23476. D.J.A. is an Investigator of the Howard Hughes Medical Institute.

Received: November 28, 2001
Revised: February 13, 2002
Published online: March 11, 2002

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Neuron, Vol. 45, 55–67, January 6, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2004.12.026

Multiple Dorsoventral Origins of Oligodendrocyte Generation in the Spinal Cord and Hindbrain

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Summary

Studies have indicated that oligodendrocytes in the spinal cord originate from a ventral progenitor domain defined by expression of the oligodendrocyte-determining bHLH proteins Olig1 and Olig2. Here, we provide evidence that progenitors in the dorsal spinal cord and hindbrain also produce oligodendrocytes and that the specification of these cells may result from a dorsal evasion of BMP signaling over time. Moreover, we show that the generation of ventral oligodendrocytes in the spinal cord depends on Nkx6.1 and Nkx6.2 function, while these homeodomain proteins in the anterior hindbrain instead suppress oligodendrocyte specification. The opposing roles for Nkx6 proteins in the spinal cord and hindbrain, in turn, appear to reflect that oligodendrocytes are produced by distinct ventral progenitor domains at these axial levels. Based on these findings, we propose that oligodendrocytes derive from several distinct positional origins and that the activation of Olig1/2 at different positions is controlled by distinct genetic programs.

Introduction

Neurons, oligodendrocytes, and astrocytes represent the three fundamental cell types of the vertebrate central nervous system (CNS), and the generation of these cell types at precise positions and specific time points during development is critical for the establishment of brain function. Insight has been obtained into the molecular mechanisms that control the generation of specific neuronal subtypes in space and time (Jessell, 2000; Lumsden and Krumlauf, 1996; Pattyn et al., 2003b). Oligodendrocytes and astrocytes are generated subsequent to neurogenesis (Rowitch, 2004), and less is known about the positional determination of these glial cell types during CNS development.

Oligodendrocytes are the myelinating cells of the CNS that insulate axons, while astrocytes provide structural support, regulate water balance, and maintain the blood-brain barrier (Rowitch, 2004). Glial cells originate from neural progenitors in the ventricular zone (VZ), and once specified they leave the VZ and migrate as proliferative precursors to occupy all regions of the CNS. Studies in the spinal cord suggest that oligodendrocytes are produced by a small group of ventral progenitors close to the floor plate, while astrocytes appear to be generated from more dorsally located progenitors (Hall et al., 1996; Lu et al., 2002; Pringle and Richardson, 1993; Zhou and Anderson, 2002). Functional analysis of the oligodendrocyte-determining basic-helix-loop-helix (bHLH) proteins Olig1 and Olig2 (collectively termed Olig1/2) support the idea that oligodendrocytes and astrocytes are generated from distinct progenitor domains and suggest further that oligodendrocytes and astrocytes are positionally specified (Lu et al., 2002; Zhou and Anderson, 2002; Zhou et al., 2001) according to strategies similar to those determining neuronal subtypes (Jessell, 2000).

In the spinal cord, different neurons emerge at specific dorsoventral (DV) positions in response to local Sonic hedgehog (Shh) signaling by ventral midline cells (Jessell, 2000) and bone morphogenetic proteins (BMPs) secreted from the dorsal midline of the neural tube (Lee et al., 2000; Liem et al., 1997). In ventral positions, graded Shh signaling controls patterning by regulating the regional expression of a set of homeodomain (HD)-containing transcriptional repressors (Briscoe et al., 2000; Muhr et al., 2001), thereby establishing a combinatorial code of HD protein expression, which defines five progenitor domains. Each domain, in turn, produces a distinct neuronal subtype (Jessell, 2000). Olig1/2 is induced by Shh, and its expression is confined to an individual ventral progenitor domain (termed pMN domain) that sequentially produces spinal motor neurons (sMNs) and oligodendrocytes (Rowitch, 2004). In *Olig1/2* mutant mice, pMN progenitors acquire an identity typical of more dorsal progenitors, and the loss of sMNs and oligodendrocytes in these mice is accompanied by a concomitant gain of V2 neurons and astrocytes (Lu et al., 2002; Zhou and Anderson, 2002). Apart from revealing an absolute requirement for Olig1/2 for oligodendrocyte generation, these data show that Olig1/2 also suppress astroglial fate in pMN progenitors (Zhou and Anderson, 2002), indicating that oligodendroglial and astroglial lineages are spatially separated in vivo at early developmental stages (reviewed in Rowitch, 2004).

While the data above demonstrate a restricted ventral origin of oligodendrocytes from pMN progenitors, it remains unclear if also other progenitors produce oligodendrocytes in vivo. In a series of quail-to-chick grafting experiments, Cameron-Curry and LeDouarin provided data suggesting that dorsal progenitors can produce oligodendrocytes (Cameron-Curry and Le Douarin, 1995). Similar experiments by Pringle and coworkers, however, instead argued that dorsal progenitors only generate astrocytes (Pringle et al., 1998). In vitro assays have further suggested the existence of a glial-restricted progenitor cell that can be derived from both dorsal and ventral parts of the spinal cord and give rise to oligodendrocytes and astrocytes in culture (Rao et al., 1998). Also, tripotent self-renewing stem cells that generate neurons, oligodendrocytes, and astrocytes in vitro can be isolated from most parts of the developing and adult CNS (Gage, 2000; Qian et al., 2000; Seaberg and van der Kooy, 2003), implicating that oligodendrocytes and astrocytes are derived from common precursors

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broadly distributed in the CNS. However, a recent study has questioned the presence of such tripotent stem cells in vivo, since the exposure of cells to fibroblast growth factor 2 (FGF2), the primary mitogen used to propagate stem cells, can result in a deregulated positional identity of neural progenitors in vitro (Gabay et al., 2003). The potency of progenitors isolated from a given position of the CNS may therefore reflect a quality acquired from exposure to FGF2 in vitro, rather than revealing their endogenous capacity in vivo. Thus, while it is well established that oligodendrocytes are produced by ventral progenitors, it is uncertain if also other regions of the developing CNS give rise to these cell types in vivo.

We have explored the positional specification of oligodendrocytes in the spinal cord and hindbrain and provide in vivo and in vitro evidence that, in addition to the ventral pMN domain, oligodendrocytes are generated from dorsal progenitors at both these axial levels. High concentrations of BMPs block the specification of dorsal Olig2$^+$ cells in vitro, and their generation is promoted when BMP signaling is inhibited, indicating that a progressive decrease of dorsal BMP signaling over time influences the temporal appearance of oligodendrocytes in the dorsal neural tube. In addition, we show that pMN domain-derived oligodendrocytes essentially are missing in the spinal cord of mice lacking the ventrally expressed HD proteins Nkx6.1 and Nkx6.2 (collectively termed Nkx6 proteins). We find that these HD proteins instead suppress oligodendrocyte production in the anterior hindbrain. These unanticipated opposite roles for Nkx6 proteins in the spinal cord and hindbrain, in turn, reflect that oligodendrocytes are produced by distinct ventral progenitor domains at these axial levels. Taken together, our data suggest that oligodendrocytes are generated from several distinct DV progenitor domains and that the activation of Olig1/2 at different positions is controlled by distinct genetic programs.

Results

A Dorsal Origin of Oligodendrocyte Generation in the Hindbrain

Similar to the spinal cord, oligodendrocytes in the hindbrain are generated from ventral progenitors, and their production depends on Shh signaling (Alberta et al., 2001) and Olig1/2 function (Lu et al., 2002; Zhou and Anderson, 2002). However, Olig1/2 expression in the hindbrain is also detected in the VZ at more dorsal positions (Figures 1A and 1B) (Liu et al., 2003), and the fate of these cells has not been determined. At E13.5, one dorsal *Olig1/2* expression domain spans along the axial extent of the hindbrain, with the exception of rhombomere 1 (Figure 1A). To begin to examine this population of Olig1/2$^+$ cells, we mapped their DV position in relation to the expression of HD proteins that define different DV progenitor domains. While ventral *Olig1*$^+$ cells at E13.5 were detected within the pMNv domain that expresses the HD protein *Nkx2.2* (see below), the dorsal Olig1$^+$ cells were located immediately dorsal to the expression domain of *Dbx2* (Pierani et al., 1999), but within the domain of *Pax3*, *Pax7*, and *Gsh1* expression (Figures 1B–1F; data not shown). Pax3, Pax7, and Gsh1 are definitive markers of dorsal progenitor cells at this axial level (Goulding et al., 1991; Jostes et al., 1990; Sander et al., 2000; Valerius et al., 1995), suggesting that this population of Olig1/2$^+$ cells is located within the alar plate. The OLP marker *Sox10* (Kuhlbrodt et al., 1998) was expressed in a fashion similar to *Olig1* in the dorsal hindbrain (Figure 1G). As determined by immunohistochemistry, several dorsal Olig2$^+$ cells in the VZ coexpressed Gsh1 (Figure 1H) while more laterally positioned, and presumably more mature, cells coexpressed the OLP markers PDGFRα (Hall et al., 1996) and NG2 (Figures 1J and 1K) (Nishiyama et al., 1996) but not the panneuronal marker NeuN (Figure 1I) (Mullen et al., 1992). Together, these data strongly suggest that at least a subset of Olig1/2$^+$ cells in the hindbrain originates from dorsal progenitors and are consistent with the idea that these cells differentiate into oligodendrocytes.

To more extensively investigate if dorsal progenitors in the hindbrain generate oligodendrocytes, we examined the capacity of hindbrain explants to generate OLPs in vitro. In this assay, explants corresponding to the ventral and dorsal Olig1/2$^+$ domains (Figure 1X) were isolated at E10.5, approximately 2–3 days before Olig1/2$^+$ OLPs can be detected in vivo (Miller, 2002). Explants isolated from tissue intervening the ventral and dorsal Olig1/2$^+$ domains (intermediate explants; Figure 1X) were included as controls, since this Olig1/2$^-$ domain is predicted not to produce oligodendrocytes in vivo or in vitro. Explants were cultured in defined media containing platelet-derived growth factor (PDGF-AA) for various time points, and OLP generation was scored by monitoring expression of the OLP markers Olig2, PDGFRα, NG2, and the O4 antigen (Sommer and Schachner, 1981). Importantly, we did not add fibroblast growth factors (FGFs) to the culture media, since FGF2 has been shown to ventralize cultured dorsal neural progenitor cells, resulting in an arbitrary in vitro-triggered induction of Olig2 expression and oligodendrocyte differentiation (Chandran et al., 2003; Gabay et al., 2003).

In ventral and dorsal explants cultured for 6 days, an extensive number of Olig2$^+$ cells were detected, and the majority of cells coexpressed PDGFRα and NG2 (Figures 1L, 1N, 1O, and 1Q). After 8–10 days in vitro, ventral explants showed significant expression of the more mature oligodendrocyte lineage marker O4 (Figure 1T). Olig2$^+$/O4$^+$ cells were detected also in dorsal explants, albeit the number of double-positive cells was lower as compared to ventral explants (Figure 1R). Importantly, in intermediate explants cultured under identical conditions, expression of Olig2, PDGFRα, or O4 was not detected at any time point analyzed (3–10 days of culture; Figures 1M, 1P, and 1S and data not shown). Thus, progenitors isolated from a DV domain that lacks expression of Olig1/2 in vivo do not generate OLPs under these in vitro culturing conditions. Given that dorsal but not the more ventral intermediate explants generate OLPs, it is unlikely that the OLPs observed in dorsal explants represent ventrally derived OLPs that at the time of tissue isolation had migrated into dorsal positions. Moreover, the absence of OLPs in intermediate explants makes it unlikely that the generation of OLPs in dorsal explants result from a deregulated, or ventralized, potential of dorsal progenitors due to the culturing conditions. In additional support for this, we could detect expression of Pax7 (Figure 1U) but not the ventral mark-

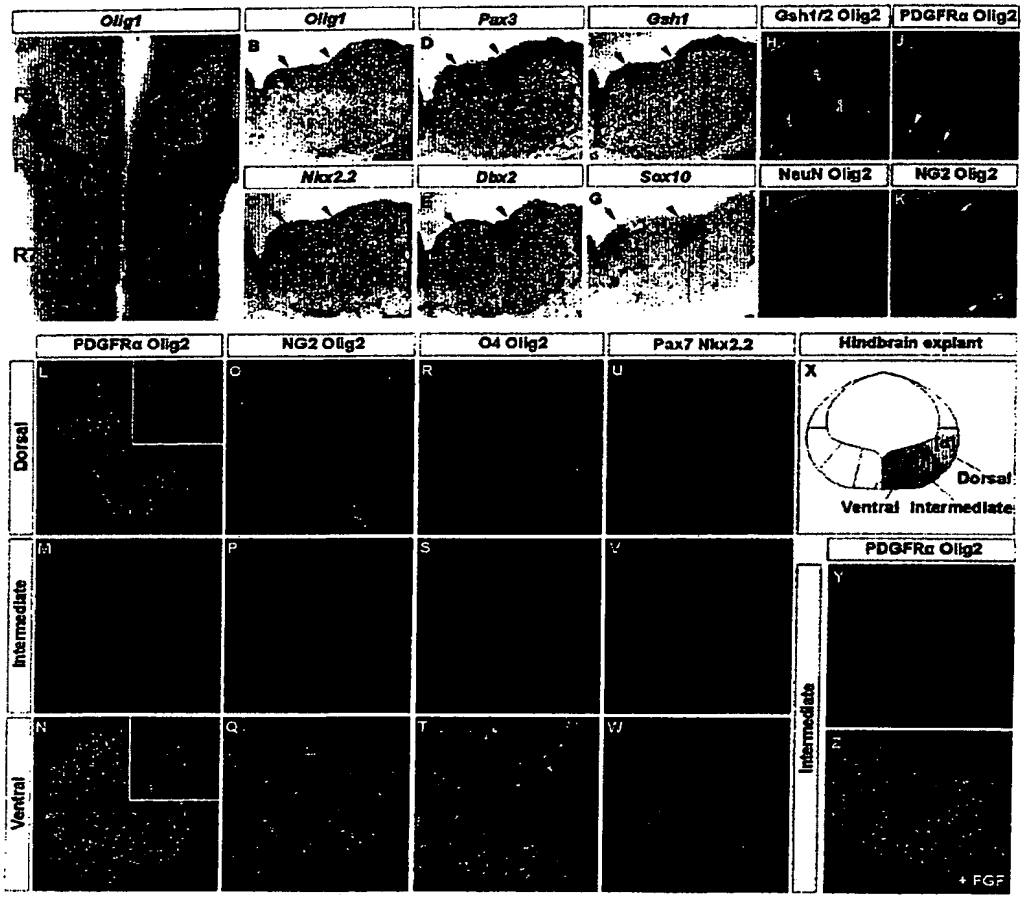

Figure 1. Dorsal Olig1/2$^+$ Progenitors in Hindbrain Give Rise to Oligodendrocytes

(A) Dorsal flat-mount view showing expression of *Olig1* in the ventral (arrow) and dorsal (arrowhead) hindbrain (HB).

(B–G) Transverse sections of rhombomere (R) 4 of the HB at E13.5 showing expression of *Olig1* (B), *Nkx2.2* (C), *Pax3* (D), *Dbx2* (E), and *Gsh1* (F). Dorsal *Olig1*$^+$ cells (ventral boundary indicated by arrowhead) are located within the domain of *Gsh1* and *Pax3*. Ventral *Olig1*$^+$ cells are detected within the *Nkx2.2*$^+$ domain (dorsal boundary indicated by arrow). *Sox10* (G) is at E12.5 expressed in a fashion similar to *Olig1* (B).

(H–K) A subset of dorsal Olig2$^+$ cells expresses Gsh1/2 (H), PDGFRα (arrows in [J]), and NG2 (arrows in [K]) but not NeuN (I).

(L–W) Ventral ([v]) and dorsal ([d]) but not intermediate ([i]) HB explants isolated at E10.5 generate oligodendrocytes in vitro. After 6 days in culture, Olig2$^+$/PDGFRα$^+$ cells are present in dorsal (inset in [L]) and ventral (inset in [N]) explants. After 8 days, Olig2$^+$ cells in ventral and dorsal explants express NG2 (O and Q) and O4 (R and T). No cells in intermediate explants expressed Olig2, PDGFRα, NG2, or O4 (M, P, and S). HB explants retain their dorsoventral identity, shown at 3 days in culture. Dorsal explants express Pax7 (U), while ventral express Nkx2.2 (W).

(X) Illustration of isolated dorsal, intermediate, and ventral explants.

(Y and Z) FGF2 induces Olig2 and PDGFRα expression in intermediate explants, shown after 8 days in culture.

ers Nkx2.2 or Shh (Figures 1U and 1W; data not shown) in dorsal explants cultured for 2–5 days.

It is possible that the absence of OLPs in intermediate explants could reflect that intermediate progenitors, in contrast to their more ventral or dorsal counterparts, have lost competence to generate oligodendrocytes at the time of tissue isolation. To examine this, we cultured intermediate explants in media enriched with FGF2. In contrast to explants cultured in PDGF-AA-enriched media without FGF2 (Figure 1Y), numerous Olig2$^+$/PDGFRα$^+$-expressing cells were detected in intermediate explants cultured in the presence of FGF2 (Figure 1Z). These data show that intermediate progenitors have the potential to produce OLPs and support the notion that addition of FGF2 to neural progenitor, or stem cell, cultures in vitro induces oligodendrocyte differentiation in cells not fated to generate these cells in vivo. Taken together, these data lend strong support to the idea that oligodendrocytes are derived from both ventral and dorsal Olig1/2$^+$ progenitor domains in the hindbrain.

Dorsal Progenitors in the Spinal Cord Generate Olig2-Expressing Cells and Give Rise to Oligodendrocytes in Culture

The generation of oligodendrocytes has been most extensively studied in the spinal cord (Rowitch, 2004). A

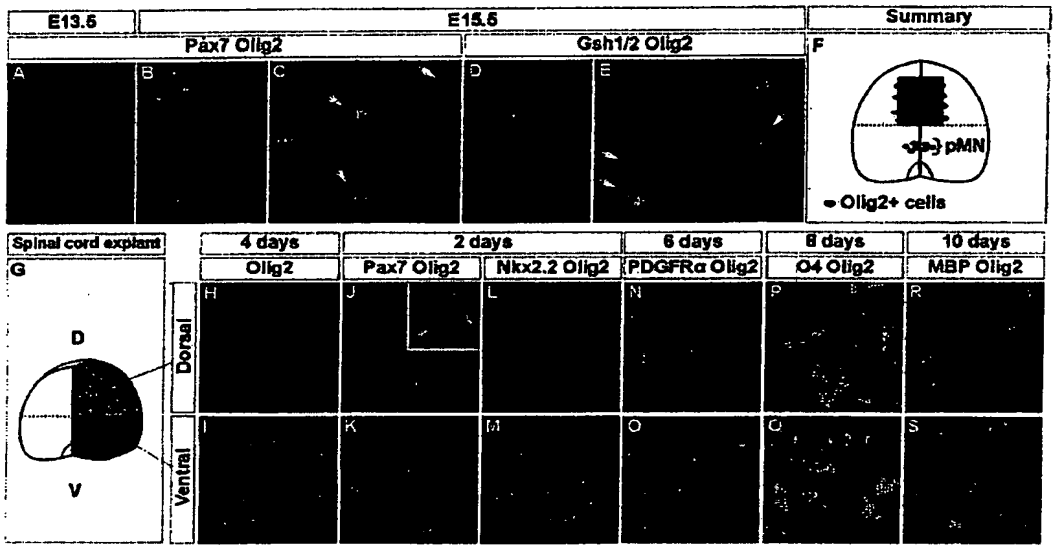

Figure 2. Dorsal Spinal Cord Progenitors Express Olig2 and Generate Oligodendrocytes In Vitro

(A–E) Transverse sections of thoracic spinal cord (SC) at E13.5 and E15.5 showing expression of Olig2 relative to Pax7 (A–C) and Gsh1/2 (D and E). Several dorsally positioned Olig2$^+$ cells coexpress Pax7 (C) and Gsh1/2 (E) at E15.5.

(F) Summary illustrating that Olig2$^+$ cells can be detected both in the ventral and dorsal ventricular zone (VZ).

(G) Illustration of the division of SC into dorsal and ventral explants.

(H–S) Whole-mount staining of dorsal and ventral explants isolated at E12.5. Cells in dorsal and ventral explants express Olig2 (H and I) and retain their dorsoventral identity after 2 days of culture; dorsal explants express Pax7 (J), and ventral explants express Nkx2.2 (M). Inset in (J) shows Olig2$^+$ cells that express Pax7. Olig2$^+$ cells in dorsal and ventral explants express PDGFRα after 6 days (N and O), O4 after 8 days (P and Q), and MBP after 10 days in vitro (R and S).

major source of oligodendrocyte production at this level is the ventral pMN domain that expresses Olig1 and Olig2 (Hall et al., 1996; Lu et al., 2002; Pringle and Richardson, 1993; Zhou and Anderson, 2002). It remains uncertain if also other progenitor domains in the spinal cord generate oligodendrocytes in vivo (Cameron-Curry and Le Douarin, 1995; Miller, 2002; Pringle et al., 1998; Richardson et al., 2000; Spassky et al., 2000). Our data suggesting a dual origin of oligodendrocytes in the hindbrain prompted us to examine the positional generation of oligodendrocytes in the spinal cord. In agreement with previous studies, the expression of Olig2 within the VZ was selectively confined to the ventral pMN domain at the peak of OLP specification at E13.5 (Figure 2A). At E15.5, migrating Olig2$^+$ OLPs were detected throughout the spinal cord (Figures 2B and 2D; data not shown). Interestingly, many Olig2$^+$ cells located within or in close proximity to the dorsal VZ coexpressed the dorsal progenitor markers Pax7 and Gsh1/2 at E15.5 (Figures 2C and 2E). Dorsal Olig2$^+$ cells located at a distance from the VZ did not express these markers (Figures 2B and 2D).

The presence of Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ in the dorsal spinal cord raised the possibility that dorsal progenitors generate Olig2$^+$ oligodendrocytes at this level. Alternatively, a subset of migrating Olig2$^+$ cells generated from the pMN domain could invade the dorsal VZ and initiate expression of Pax7 and Gsh1/2 at E15. To distinguish between these possibilities, we compared the ability of isolated ventral and dorsal spinal cord explants to generate oligodendrocytes in vitro. In these experiments, ventral and dorsal explants were isolated at E10.5 or at E12 and thus prior to any dorsal migration of pMN-derived OLPs (Sussman et al., 2000). Explants were cultured in media containing PDGF-AA but not FGF2 for various time points. In these conditions, OLP differentiation was observed in both ventral and dorsal explants after 4–8 days of culture, as determined by Olig2$^+$ cells that coexpressed PDGFRα, NG2, and the O4 antigen (Figures 2N–2Q; data not shown). After 8–10 days, Olig2-expressing cells in ventral and dorsal explants had initiated expression of myelin basic protein (MBP), a marker of mature oligodendrocytes (Figures 2R and 2S) (Lemke, 1988). Similar results were obtained from ventral and dorsal spinal cord explants isolated from E10.5 and E12.5 embryos (data not shown).

We next examined the DV identity of cells in spinal cord dorsal and ventral explants. In dorsal explants isolated at E12, Olig2$^+$ cells could first be detected after 2–3 days of culture, a time point that corresponds well with the appearance of Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ cells at around E15 in vivo (Figures 2J, 2L, and 2B–2E; data not shown). At these stages, Pax7 expression was observed while no expression of ventral markers Nkx2.2, Nkx6.1, or Shh could be detected (Figures 2J and 2L; data not shown). Importantly, several Olig2$^+$ cells in dorsal explants coexpressed Pax7 (Figure 2J). Similar results were obtained when dorsal explants were isolated at E10.5 (data not shown). In ventral explants, expression of Nkx2.2, Nkx6.1, and Shh but not Pax7 could be de-

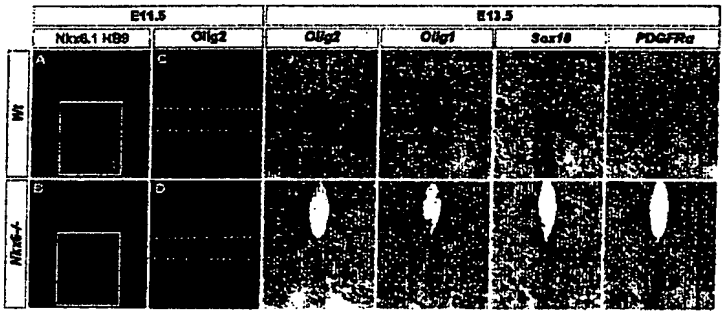

Figure 3. A Loss of Ventrally Derived Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

(A–L) Transverse thoracic SC sections of wild-type (wt) and *Nkx6* mutant mice at E11.5 and E13.5. Expression of Nkx6.1 and the MN marker Hb9 in wt (A) and *Nkx6* mutants (B) at E11.5. Box in (A) and (B) marks part of SC shown in (C)–(L). The expression of Olig2 in the pMN domain is lost (C and D). At E13.5, expression of the OLP markers Olig2 (E and F), *Olig1* (G and H), *Sox10* (I and J), and *PDGFRα* (K and L) are missing in the ventral spinal cord of *Nkx6* mutant mice. Dotted lines in (C)–(L) indicate pMN domain boundaries.

tected (Figures 2K and 2M; data not shown). These data show that dorsal Pax7$^+$ progenitors generate Olig2-expressing cells and that Olig2$^+$ cells differentiate along the OLP lineage in vitro and argue against the possibility that Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ detected in vivo would represent dorsally migrating cells that originate from the pMN domain. The retained DV identity of cells in dorsal explants also seems to exclude the possibility that OLPs in dorsal explants are generated in response to an in vitro-induced, albeit FGF-independent, ventralization of progenitor cell identity.

A Loss of Ventrally Derived Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

To further examine the possibility of a dual ventral and dorsal origin of oligodendrocytes in the spinal cord, we analyzed mice lacking the related HD proteins Nkx6.1 and Nkx6.2. Nkx6 proteins are expressed in the ventral neural tube, including the pMN domain, and their function is necessary for the ventral expression of Olig2 and the generation of MNs in the spinal cord (Figures 3A and 3B) (Novitch et al., 2001; Vallstedt et al., 2001). The generation of oligodendrocytes in the spinal cord has not been examined in *Nkx6* mutants, but the ventral extinction of Olig2 expression raised the possibility that pMN domain-derived oligodendrocytes may be affected. In agreement with this, we could not detect any expression of *Olig1* or Olig2 in the ventral spinal cord between E11.5 and E13.5 (Figures 3D, 3F, and 3H), the time at which ventral oligodendrocytes are being specified (Hall et al., 1996). Also, while *Sox10* and *PGDFRα* could be detected in the pMN domain and/or in migrating OLPs in controls at E13.5, the expression of these OLP markers was missing in *Nkx6* mutants (Figures 3I–3L). These data show that Nkx6 proteins are required not only for the generation of spinal MNs, but also for the subsequent specification of oligodendrocytes from the pMN domain.

Oligodendrocytes Are Generated from Progenitors with a Dorsal Identity in the Spinal Cord of *Nkx6* Mutants

Olig1/2 is required for the generation of all oligodendrocytes regardless of their developmental origin in the CNS (Lu et al., 2002; Zhou and Anderson, 2002). Our data indicate that Nkx6 proteins are necessary for the generation of pMN domain-derived oligodendrocytes in the spinal cord. However, since Nkx6.1 and Nkx6.2 are expressed only in ventral progenitors, they are not predicted to affect the generation of putative dorsally derived oligodendrocytes. We therefore examined the generation of oligodendrocytes in *Nkx6* mutants at E15.5, a stage when Olig2$^+$ cells that coexpress Pax7 and Gsh1/2 can be detected in the dorsal spinal cord in wild-type embryos (Figures 2C and 2E). In controls at this stage, OLPs were evenly distributed in the spinal cord gray matter, as determined by *Olig1* expression (Figure 4C). Strikingly, numerous *Olig1*-expressing cells were observed also in *Nkx6* mutants, but in contrast to controls, essentially all *Olig1*$^+$ cells were located in the dorsal half of the spinal cord (Figure 4D). The number and distribution of Olig2$^+$ cells coexpressing Pax7 and/or Gsh1/2 in lateral positions of the dorsal VZ was similar in mutants and controls at E15 (Figures 4E–4J and 4W). These data provide strong genetic evidence that Olig1/2$^+$ cells are generated from the dorsal VZ in vivo. A few Olig1/2$^+$cells could occasionally be detected in ventral positions at E15 (Figure 4F). Therefore, we cannot exclude the possibility that a subset of Olig1/2$^+$ cells in *Nkx6* mutants are generated also from ventral progenitors.

In control embryos at E15, a subset of Olig2$^+$ cells in the dorsal spinal cord expressed PDGFRα, while only rare Olig2$^+$/PDGFRα$^+$ cells could be detected in *Nkx6* mutants at this stage (Figures 4K and 4L). We noticed that a population of Olig2$^+$ cells that lacked the expression of PDGFRα and NG2 was present at E15.5 in wild-type mice, and this population corresponded in number to the Olig2+/PDGFRα$^-$/NG2$^-$ cells observed in mutant mice (Figure 4X; data not shown). At E18.5, Olig2$^+$ cells were evenly distributed in *Nkx6* mutants (Figure 4N), and the majority of these cells had at this stage initiated expression of PDGFRα and NG2 (Figures 4P, 4R, and 4X). Consistent with the generation of oligodendrocytes from dorsal progenitors in vitro, these data provide genetic evidence that Olig1/2$^+$ cells with a dorsal origin acquire molecular characteristics of OLPs also in vivo. Additionally, the uniform distribution of Olig1/2 cells in *Nkx6* mutants at E18.5 indicates that dorsally derived OLPs migrate into the ventral neural tube, at least in conditions when the generation of pMN domain-derived oligodendrocytes is compromised. We could not examine if OLPs in *Nkx6* mutants acquire a terminal oligodendrocyte phenotype in vivo, since *Nkx6* mutant embryos die at birth, the time when OLPs begin to terminally

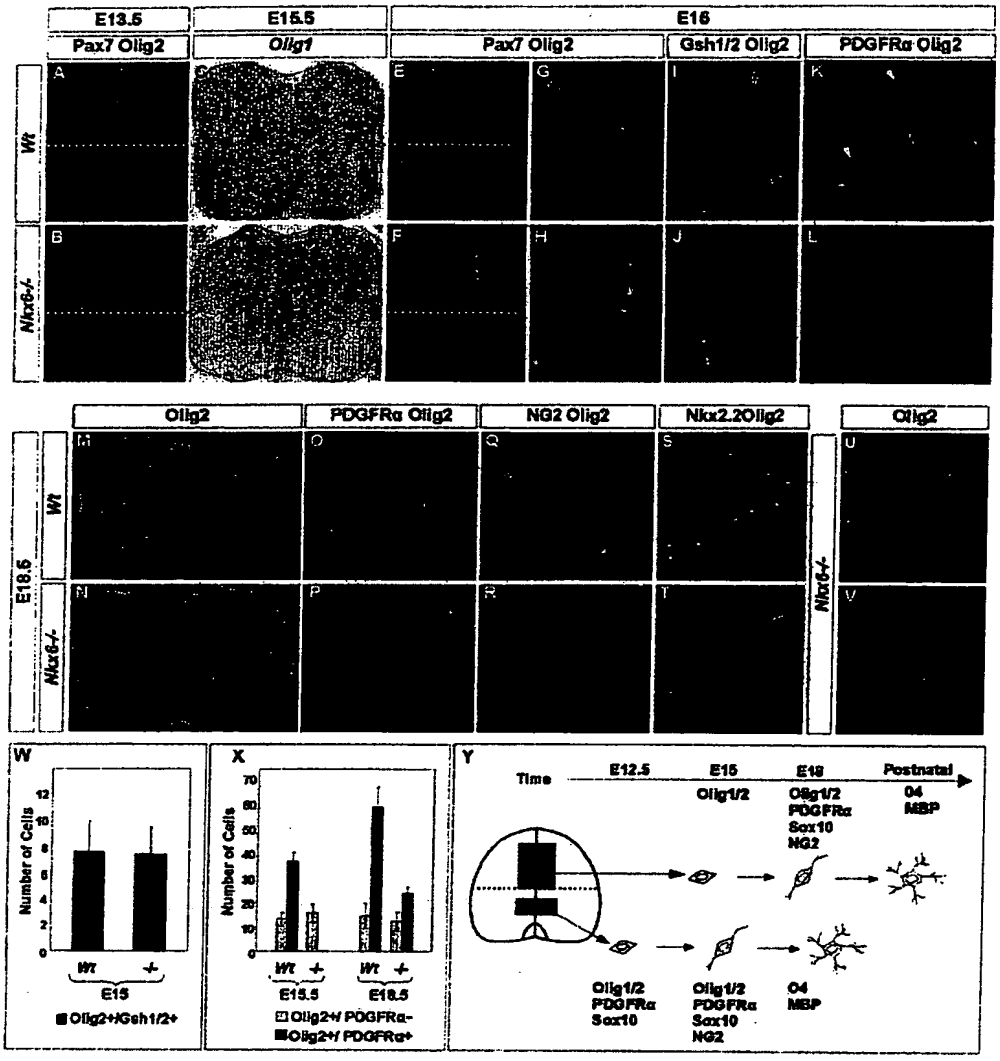

Figure 4. Dorsal Olig1/2$^+$ Cells Generate Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

(A–L) Transverse thoracic SC sections of wt and *Nkx6* mutant embryos at E13.5 to E15.5. Ventral Olig2$^+$ cells are detected in controls (A) but not *Nkx6* mutants (B) at E13.5. At E15.5, *Olig1* expression is detected throughout the SC of controls (C). In *Nkx6* mutants, *Olig1* expression at E15.5 is detected predominantly in the dorsal SC (D). Dorsal Olig2$^+$ cells in controls and *Nkx6* mutants at E15.5 coexpress Pax7 (E–H) and Gsh1/2 (I and J). At E15.5, a small number of Olig2$^+$ cells (one to two cells per section) was also detected within, or in close proximity to, the ventral VZ in *Nkx6* mutants (F). Dotted line in (A)–(F) indicates the ventral boundary of Pax7 expression. Olig2$^+$ cells in *Nkx6* mutants do not express PDGFRα at E15.5 (L), while both Olig2$^+$/PDGFRα$^-$ cells (arrow in [K]) and Olig2$^+$/PDGFRα$^+$ cells (arrowhead in [K]) are found in controls at this stage.

(M–T) Transverse thoracic SC sections of wt and *Nkx6* mutant mice at E18.5. Olig2$^+$ cells are evenly distributed along the DV axis in wt (M) and *Nkx6* mutants (N), and the majority of cells express PDGFRα (O and P) and NG2 (Q and R). Olig2$^+$/Nkx2.2$^+$ are detected in wt (S) but not *Nkx6* mutant embryos (T).

(U and V) *Nkx6* mutant SC tissue isolated at E12.5 and cultured for 10 days generate Olig2$^+$ cells that coexpress O4 (U) and MBP (V).

(W) Quantification of Olig2$^+$/Gsh1/2$^+$ cells in wt and *Nkx6* mutants at E15.5. Counts from six to eight sections per embryo; n = 2 wt and 3 *Nkx6* mutants; mean ± SD.

(X) Quantification of Olig2$^+$/PDGFRα$^-$ and Olig2$^+$/PDGFRα$^+$ in the SC of wt and *Nkx6* mutant embryos at E15.5 and E18.5. Similar numbers of Olig2$^+$/PDGFRα$^-$ are present in wt and mutant mice at both time points. Counts from ten sections; mean ± SD.

(Y) Model indicating the differentiation profile of ventral and dorsal oligodendrocytes in the SC.

differentiate (Baumann and Pham-Dinh, 2001). Nevertheless, Olig2$^+$ cells that expressed O4 and MBP could be detected in isolated *Nkx6* mutant spinal cord tissue after culture in vitro (Figures 4U and 4V), indicating that these cells have the capacity to differentiate into mature oligodendrocytes.

Dorsal and Ventral OLP Lineages Express Distinct Molecular Properties at Prenatal Stages

Are oligodendrocytes generated from distinct positional origins molecularly and functionally equivalent? Studies of neuronal differentiation suggest that neurons with similar functional properties have certain common molecular properties, but also that functional differences among cells within a given class are associated with subtype-specific profiles of gene expression (Jessell, 2000). As compared to neuronal cell differentiation, little is known about the determination of different glial subtypes (Rowitch, 2004). To examine the molecular properties of dorsally and ventrally derived oligodendrocytes, we compared the gene expression profile of Olig2$^+$ OLPs in controls and *Nkx6* mutant mice at E18.5. In controls, a subset of Olig2$^+$/PDGFRα$^+$ cells in the spinal cord had initiated expression of Nkx2.2 (29% ± 2.5%) (Figure 4S) (Qi et al., 2001). In contrast, few if any Olig2$^+$/PDGFRα$^+$ cells in *Nkx6* mutants expressed Nkx2.2 at this stage (Figure 4T). The lack of OLPs that express Nkx2.2 in *Nkx6* mutants indicate that Nkx2.2 selectively marks OLPs with a ventral origin and that OLPs with different origins are molecularly distinct, at least at prenatal stages of spinal cord development.

Evasion of BMP Signals Influences the Timing of Olig1/2 Induction in Dorsal Progenitors

The occurrence of dorsally generated oligodendrocytes raises the issue of how these cells are specified. In the neural tube, local BMP signaling has a central role in the initial establishment of dorsal progenitor identity (Lee et al., 2000; Liem et al., 1997; Nguyen et al., 2000). BMPs expressed in the dorsal neural tube also function to suppress more ventral Shh-dependent cell fates, including MNs (Liem et al., 2000) and ventral oligodendrocytes derived from the pMN domain (Hall and Miller, 2004; Mekki-Dauriac et al., 2002). The activation of Olig1/2 expression in dorsal progenitors occurs at around E15, a stage when the neural tube has grown considerably in size. We therefore considered the possibility that dorsally generated oligodendrocytes could be sensitive to BMPs and that their late birth could reflect a decrease of BMP signaling over time. To examine this, we analyzed the number of Olig2$^+$ cells in dorsal explants that were isolated at E10.5 or E12.5 and exposed to BMP7 or the BMP antagonists Noggin and Chordin (Piccolo et al., 1996; Zimmerman et al., 1996). In dorsal explants isolated at E10.5, the generation of Olig2$^+$ cells was completely blocked when cells were exposed to 1 ng/ml of BMP7 (Figure 5G; data not shown). Interestingly, the exposure of E10.5 dorsal explants to Noggin/Chordin resulted in a 3- to 4-fold increase in the number of Olig2$^+$ cells as compared to controls (Figures 5A, 5B, and 5E). This increase did not reflect a ventralization of progenitor identity, as indicated by the in vitro detection of Olig2$^+$/Pax7$^+$ cells (Figure 5F) and a lack of any detectable induction of Nkx2.2 or Nkx6.1 expression (data not shown). Instead, the increased number of Olig2$^+$ cells in E10.5 explants exposed to Noggin/Chordin correlated with a more rapid induction of these cells as compared to controls (Figures 5H–5L). BMP7 blocked Olig2 expression also in dorsal explants isolated 2 days later at E12.5 (data not shown). In contrast to explants isolated at E10.5, however, exposure of E12.5 explants to Noggin and Chordin did not result in an increased generation of Olig2$^+$ cells (Figures 5C–5E). While these data show that dorsal progenitors are still responsive to BMPs at E12.5, the failure of BMP antagonists to promote the generation of Olig2$^+$ cells at later stages is consistent with the idea that concentration of BMPs in the dorsal neural tube decreases over time.

Oligodendrocytes Derive from Different Ventral Progenitor Domains in the Spinal Cord and Hindbrain

While Nkx6 proteins are required for Olig2 expression in the ventral spinal cord, the expression of Olig2 persists in the ventral hindbrain of *Nkx6* mutants and is even ectopically activated at anterior hindbrain levels (Pattyn et al., 2003b). The differential regulation of Olig2 in the spinal cord and hindbrain raised the possibility that the generation of ventral oligodendrocytes is regulated differently at these axial levels. In support for this, and in contrast to the spinal cord, we found an extensive ventral ectopic induction of *Olig1*, Olig2, *Sox 10*, and PGDFRα in the anterior hindbrain of *Nkx6* mutants at E12.5 as compared to controls (Figures 6H, 6J, 6L, and 6N and data not shown). These data provide direct evidence that Nkx6 proteins suppress oligodendrocyte generation in ventral positions of the anterior hindbrain. Dorsally derived OLPs in the hindbrain, however, appeared to be unaffected by the loss of Nkx6 function (Figures 6G and 6H).

How then can Nkx6 proteins mediate opposing effects on the generation of oligodendrocytes at different axial levels of the CNS? Most HD transcription factors that are involved in ventral neural patterning, including Nkx6.1 and Nkx6.2, function directly as transcriptional repressors (Muhr et al., 2001; Novitch et al., 2001; Zhou et al., 2001). These data suggest that the promotion of Olig2 expression by Nkx6 proteins in the spinal cord is indirect and possibly involves an Nkx6-mediated exclusion of a repressor of Olig genes in pMN progenitors. In the spinal cord, the Olig1/2$^+$ pMN domain is located immediately dorsal to p3 progenitors, which express Nkx2.2 and produce *Sim1*-expressing V3 neurons (Briscoe et al., 1999). Nkx2.2 is sufficient and required for the generation of V3 neurons in the spinal cord and is an established repressor of Olig2 expression at this axial level (Muhr et al., 2001; Novitch et al., 2001; Zhou et al., 2001). Unexpectedly, we found that the domain of Nkx2.2 and *Sim1* expression had expanded dorsally and encroached into the presumptive pMN domain in the spinal cord of *Nkx6* mutants at E11.5 (Figures 6A–6D). These data show a genetic requirement for Nkx6 proteins to suppress Nkx2.2 expression in the pMN domain. Further, they reveal a strong correlation between the loss of Olig2 expression, and subsequent oligodendrogenesis, and the dorsal expansion of Nkx2.2 into the pMN domain of *Nkx6* mutant spinal cord.

The observation that loss of Nkx6 proteins in the anterior hindbrain instead results in an ectopic induction of OLP markers, led us to examine the generation of OLPS at this level in more detail. In the anterior hindbrain, ventral expression of Olig2 could first be detected at E12.5 (Figures 6E, 6I, and 6K). Most, if not all, Olig2$^+$

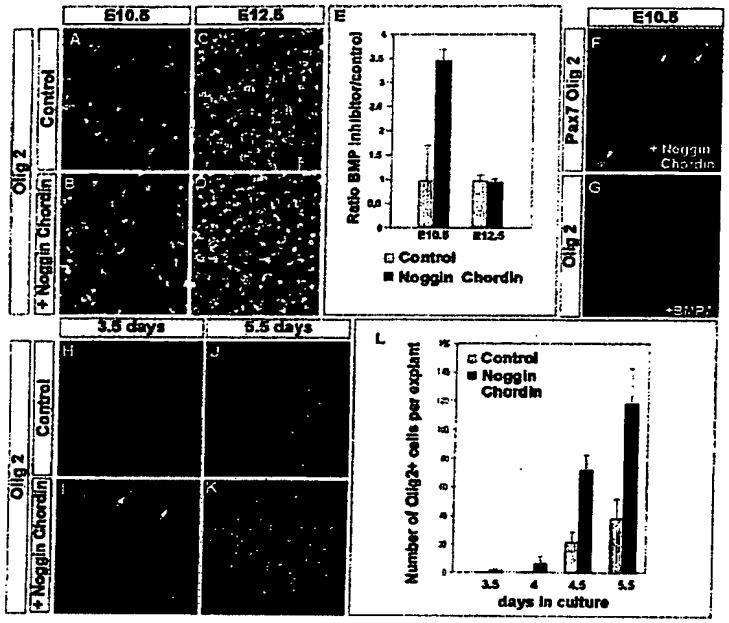

Figure 6. Decreased BMP Signaling Promotes the Generation of Dorsal Olig2⁺ Cells In Vitro

(A–D) Olig2⁺ cells in dorsal SC explants from E10.5 (A and B) and E12.5 (C and D) embryos cultured until corresponding day E18.5 in the absence (A and C) or presence (B and D) of BMP inhibitors Noggin and Chordin.

(E) Exposure of E10.5 explants to Noggin/Chordin resulted in a 3.5-fold increase in Olig2⁺ cells as compared to controls. A similar number of Olig2⁺ cells was observed in E12.5 explants cultured with or without Noggin/Chordin. Graph shows ratio of Olig2⁺ cells in Noggin/Chordin-treated explants versus controls. Eight explants analyzed per time point; mean ± SD.

(F) Explants retain their dorsal identity in the presence of Noggin/Chordin as indicated by Pax7 expression and the detection of Pax7⁺/Olig2⁺ cells (arrows in [F]).

(G) BMP7 suppresses Olig2 expression in E10.5 dorsal SC explants cultured for 8 days.

(H–K) Olig2 expression in E10.5 dorsal explants cultured for various time points in the presence or absence of Noggin/Chordin.

(L) Quantification of Olig2⁺ cells per explant. In explants exposed to Noggin/Chordin, Olig2⁺ cells could first be detected after 3.5 days of culture, as compared to 4.5 days in controls. Explants treated with Noggin/Chordin show higher numbers of Olig2⁺ cells compared to controls. Counts from six explants per time point; mean ± SD.

cells at this stage were located in lateral positions of the Nkx2.2⁺ VZ, and many cells coexpressed Nkx2.2 and Olig2 (Figures 6E and 6I). Many Olig2⁺ cells also expressed PGDFRα, indicating that they indeed are OLPs (Figure 6K). Thus, while Olig2⁺ cells in the ventral spinal cord are generated dorsal to the Nkx2.2⁺ progenitor domain, Olig1/2⁺ cells in the anterior hindbrain appear to be derived from Nkx2.2-expressing progenitors. Like spinal cord levels, the domain of Nkx2.2 expression was expanded dorsally in the anterior hindbrain of *Nkx6* mutants at E12.5 (Figures 6F and 6J; data not shown). At this axial level, however, there was a striking correlation between the expanded expression of Nkx2.2 and the ectopic induction of oligodendrocyte differentiation (Figures 6J, 6L, and 6N). These data show that the differential regulation of oligodendrocytes by Nkx6 proteins is tightly linked to the expression of Nkx2.2 and to the distinct ventral origins of oligodendrocytes in the spinal cord and anterior hindbrain (Figures 6O and 6Q).

Discussion

Previous studies have established that oligodendrocytes in the spinal cord are generated from ventral pMN progenitors in the spinal cord. In addition to a ventral origin of these cells, we here provide evidence that oligodendrocytes are produced also by progenitors in the dorsal spinal cord and hindbrain. Our study further suggests that most ventrally generated oligodendrocytes in the hindbrain are produced from Nkx2.2⁺ progenitors, rather than from more dorsally positioned pMN progenitors. Together, these data reveal multiple positional origins of oligodendrocyte specification in the spinal cord and hindbrain and provide evidence that the activation of Olig1/2 expression at different positions is regulated by distinct genetic programs.

A Dual Ventral and Dorsal Origin of Oligodendrocyte Generation in the Spinal Cord

sMNs and oligodendrocytes are generated sequentially from pMN progenitors in the ventral spinal cord, and the generation of these cells depends on the function of Olig1/2 (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). We provide several lines of evidence that, in addition to those in the pMN domain, dorsal progenitors in the spinal cord generate oligodendrocytes. First, at E15, approximately 2 days after the generation of OLPs from the pMN domain, we find that a subset of dorsal Olig2⁺ cells are located within the VZ and coexpress the established dorsal progenitor markers Pax7 and Gsh1/2 (Figures 2B–2E). Second, oligodendrocytes are produced by isolated dorsal progenitors in culture, under in vitro conditions in which cells retain their dorsal progenitor identity. Olig2⁺/Pax7⁺ cells were generated in dorsal explants isolated as early as E10.5 and, together with our analysis of *Nkx6* mutants, these experiments argue against the formal possibility that a subset of pMN domain-derived OLPs would initiate Pax7 and Gsh1 expression after migrating into the dorsal neural tube. Third, while the pMN domain and ventral oligodendrocytes are missing in the spinal cord of *Nkx6* mutants, dorsal Olig2⁺/Pax7⁺/Gsh1/2⁻ are generated on schedule and in numbers similar to those detected in controls at E15.

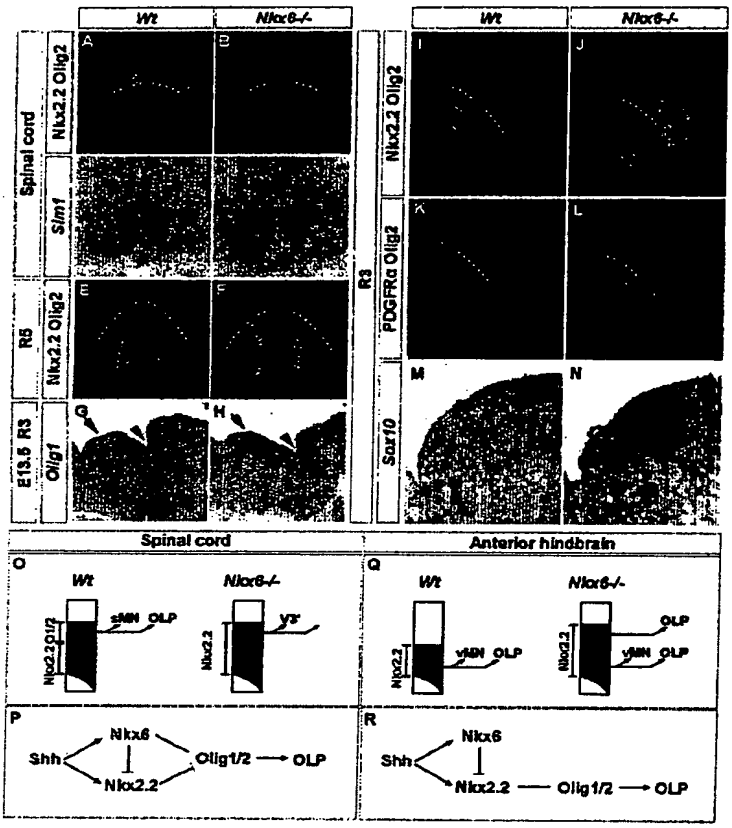

Figure 6. Different Ventral Progenitor Domains in Spinal Cord and Hindbrain Give Rise to Oligodendrocytes

(A–N) Transverse HB and SC sections of wt and *Nkx6* mutant embryos at E12.5–13.5. In the SC of wt mice, Olig2$^+$ cells are detected dorsal to the domain of Nkx2.2 expression at E12.5 (A). In *Nkx6* mutant mice, Olig2$^+$ cells are absent, and the expression of Nkx2.2 (B) and the V3 neuron marker *Sim1* (C and D) is dorsally expanded. Ventral Olig2$^+$ cells at the R5 and R3 levels of the E12.5 hindbrain are detected within, or in close proximity to, the Nkx2.2$^+$ domain (E and I). Note that several laterally positioned Nkx2.2$^+$ cells coexpress Olig2. In the *Nkx6* mutant HB, the domain of Nkx2.2 expression expands dorsally (F and J), and here the expansion is associated with an increased number and a dorsal expansion of Olig2$^+$ cells (F, J, K, and L). The generation of ectopic Olig2$^+$ cells in the ventral hindbrain of *Nkx6* mutants is accompanied by induction of OLP markers *Olig1* (G and H), PDGFRα (K and L), and *Sox10* (M and N). Dotted lines indicate dorsal boundary of wt Nkx2.2 expression. Arrow and arrowhead indicate ventral and dorsal Olig1+ domains, respectively (G and H). (O–R) Model of oligodendrocyte specification in the ventral spinal cord and hindbrain. In the ventral spinal cord and caudal-most hindbrain, somatic MNs and OLPs are sequentially generated from the Olig1/2$^+$ pMN domain located dorsal to Nkx2.2$^+$ progenitors (O). In the spinal cord, Shh induces expression of Nkx6 and Olig1/2 proteins in the pMN domain, while higher Shh concentrations induce Nkx2.2 expression in more ventral positions (P). Nkx6 proteins suppress Nkx2.2 expression in the pMN domain. The dorsal expansion of Nkx2.2 observed in *Nkx6* mutants may underlie the extinction of Olig1/2 expression in SC pMN progenitors and the ectopic generation of V3 neurons. Since Nkx6 proteins are coexpressed with Nkx2.2 in p3 progenitors, the repression of Nkx2.2 by Nkx6 is likely to require a pMN domain expressed cofactor. At anterior hindbrain levels, ventrally derived oligodendrocytes are generated from the Nkx2.2$^+$ pMNv domain that at earlier stages produce visceral MNs (Q and R). Nkx6 proteins control the dorsal limit of Nkx2.2 expression also in the hindbrain. Since Olig1/2-expressing OLPs derive from the Nkx2.2$^+$ progenitor at this level, the expansion of Nkx2.2 expression in the absence of Nkx6 function therefore results in a premature and ectopic initiation of Olig1/2 expression and oligodendrocyte differentiation (Q).

We cannot formally establish that dorsal Olig2$^+$ cells acquire properties of mature oligodendrocytes in vivo, since *Nkx6* mutants die at birth and, in wild-type embryos, differentiating dorsal Olig2$^+$ cells downregulate the expression of Pax7 and Gsh1/2 and intermingle with OLPs generated from the pMN domain. Nevertheless, our data show that cells in *Nkx6* mutants differentiate along the oligodendrocyte lineage in vivo and further that mature oligodendrocytes are produced in vitro in both cultured *Nkx6* mutant tissue and wild-type dorsal spinal cord explants. Taken together, these data strongly suggest that oligodendrocytes in the spinal cord are generated from both ventral and dorsal progenitor cells. Our study also suggests that oligodendrocytes are generated from dorsal progenitors in the hindbrain, and other studies have indicated a dual origin of oligodendrocytes in the forebrain (Gorski et al., 2002; Levison and Goldman, 1993). It is possible, therefore, that the specification of oligodendrocytes from dual, or multiple, DV positions is a general characteristic of the developing CNS.

The relative contribution of the dorsal lineage of OLPs is unclear, but comparisons of the total number of Olig1/2-expressing cells in control and *Nkx6* mutant embryos imply that dorsally generated cells represent a minor fraction of the total number of OLPs at prenatal stages of development (20%–30%; data not shown). In wild-type conditions, however, this number could be lower, since dorsally specified OLPs in *Nkx6* mutants could be propagated more efficiently due to the lack of ventrally derived OLPs that are likely to compete for essential growth factors such as PDGF (Calver et al., 1998). Also, while we observe similar numbers of Olig2$^+$ cells expressing dorsal progenitor markers in *Nkx6* mutant and controls at E15, the accompanying paper by Cai et al. (2005) in this issue of *Neuron* reports an approximately 3-fold increase in the number of Olig2$^+$/Pax7$^+$ cells in *Nkx6* mutants. While the reason for this difference between our studies remains unclear, it raises the possibility that the ventral loss of Nkx6 proteins and/or ventral oligodendrocytes may have a certain influence on the specification of OLPs from dorsal progenitor cells.

How then is the specification of dorsally derived oligo-

dendrocytes regulated? Olig1/2 proteins are required for the generation of all oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002), and a key step, therefore, must be to regulate the initiation of Olig1/2 expression in dorsal progenitor cells. Local BMP signaling from the roof plate has an essential early role in establishing dorsal neural tube identity and the patterned generation of dorsal neuronal subtypes (Helms and Johnson, 2003; Lee et al., 2000; Liem et al., 1997). Scattered Olig2$^+$/Pax7$^+$Gsh1/2$^+$ cells can first be detected in lateral positions of the VZ at around E15 (Figures 2B–2E, 4E, and 4G). We find that BMP7 suppresses oligodendrocyte differentiation in dorsal neural tube explants in vitro, while BMP antagonists enhance generation of dorsal Olig2$^+$ cells in dorsal explants isolated at early developmental stages. Since the spinal cord has grown considerably in size by E15, these data are consistent with a model in which the timing of Olig1/2 induction in dorsal progenitors involves a progressive evasion of BMP signaling due to a limited range of action of BMP signals secreted by the roof plate.

The induction of Olig1/2 expression in the pMN domain requires Shh signaling, raising the possibility that Shh also mediates the induction of Olig1/2 expression in the dorsal neural tube. This does not appear to be the case, since data by Cai and coworkers show that dorsal oligodendrocytes are generated in the absence of Shh signal transduction in vivo (Cai et al., 2005). FGF has been shown to promote Olig gene expression and oligodendrocyte differentiation in vitro (Chandran et al., 2003; Kessaris et al., 2004), but such experiments are difficult to interpret, since the induction of oligodendrocytes in response to FGFs in vitro has also been associated with an erroneous ventralization of progenitor cell identity (Gabay et al., 2003). To overcome this issue, we exposed isolated dorsal Pax7$^+$ progenitors to an inhibitor of FGF receptor signaling, SU 5402 (Mohammadi et al., 1997), and under these conditions we observed a complete block of Olig1/2 induction and oligodendrocyte differentiation (data not shown). While additional in vivo experiments are necessary to determine the precise role for BMP and FGF signaling in this process, these data indicate that a combination of FGF signaling and a progressive decrease in BMP activity over time may underlie the late phase of oligodendrocyte specification in the dorsal half of the spinal cord.

What is then the functional rationale of producing oligodendrocytes at several DV positions? One possibility is that a single origin of oligodendrocyte specification is not sufficient to produce the number of oligodendrocytes necessary to effectively insulate all axons at a given axial level. An alternative possibility is that the production of dorsal and ventral oligodendrocytes is necessary due to the establishment of physical or molecular barriers that hamper the migration of OLPs along the DV axis. Both these alternatives, however, appear to be unlikely since OLPs, once specified, are effectively propagated in a PDGF-dependent fashion outside of the VZ (Calver et al., 1998), and both ventral and dorsal OLPs appear to be capable of freely migrating along the DV axis of the spinal cord (Rowitch, 2004) (this study). The lack of Nkx2.2-expressing OLPs in *Nkx6* mutants implies that ventrally and dorsally derived OLPs express distinct molecular properties, at least at prenatal stages of development. An intriguing possibility, therefore, is that oligodendrocytes that are generated from distinct progenitor populations acquire distinct functional properties. Analyses of Olig1/2 function directly support that patterning along the DV axis controls the spatial specification of distinct glial cells, since the loss of oligodendrocytes in the pMN domain in *Olig1/2* mutants is associated with a concomitant gain of astrocytes (Zhou and Anderson, 2002). Also, in addition to the population of myelinating oligodendrocytes, certain oligodendrocytes have been shown to establish synapses with GABAergic interneurons in the hippocampus (Lin and Bergles, 2004). Clonal analyses in the postnatal forebrain have further revealed the presence of at least two types of OLPs, one that rapidly differentiates into myelinating oligodendrocytes and another that remains undifferentiated over extensive periods of time (Zerlin et al., 2004). While the mechanism(s) that underlies these functional, or behavioral, differences among oligodendrocytes remains to be determined, it is feasible that such differences will be related to the positional specification of oligodendrocytes along the DV axis of the neural tube.

Opposing Requirements for Nkx6 Proteins for Oligodendrocyte Specification in the Ventral Spinal Cord and Hindbrain

In addition to the identification of dorsally derived Olig2$^+$ OLPs in the spinal cord and hindbrain, our study reveals a striking difference between the specification of oligodendrocytes in the ventral spinal cord and hindbrain. The activity of Nkx6 proteins is required for the generation of oligodendrocytes from the ventral progenitors in the spinal cord, while the same proteins instead suppress oligodendrocyte production at anterior hindbrain levels (Figures 6O and 6Q). Our data indicate that this differential regulation reflects that oligodendrocytes derive from distinct ventral progenitor domains at these different axial levels. In the spinal cord, the pMN domain is located immediately dorsal to the Nkx2.2$^+$ p3 progenitor domain (Briscoe et al., 1999), and Nkx2.2 is an established repressor of Olig2 expression at this level (Novitch et al., 2001). A common phenotype in the spinal cord and hindbrain of *Nkx6* mutants is that the domain of Nkx2.2 expression expands dorsally, revealing a genetic requirement for Nkx6 proteins to control the dorsal limit of Nkx2.2 expression. It is conceivable, therefore, that a primary role of Nkx6 proteins in oligodendrogenesis in the ventral spinal cord is to indirectly ensure the maintained expression of Olig1/2 through the suppression of Nkx2.2.

Our analysis indicates that, in contrast to the spinal cord, the ventral oligodendrocytes in the anterior hindbrain are generated from the Nkx2.2$^+$ pMNv domain (Figures 6I and 6K) that at preceding stages has produced visceral MNs and serotonergic projection neurons (Ericson et al., 1997; Pattyn et al., 2003a, 2003b). As a consequence, the dorsal expansion of Nkx2.2 expression in the hindbrain of *Nkx6* mutants results in an increased production of OLPs rather than a loss of these cells. It remains unclear how Nkx2.2 can promote Olig1/2 expression in the hindbrain and why Nkx2.2 and Olig1/2 are coexpressed in hindbrain progenitors but not in the mouse spinal cord. Nevertheless, the expression of

Nkx2.2 at anterior hindbrain levels shows a mutually exclusive relationship with the expression of Irx3 (Pattyn et al., 2003b), a HD protein also known to repress Olig2 expression in the spinal cord (Novitch et al., 2001). Nkx2.2 could therefore promote Olig1/2 expression, at least in part, through the exclusion of Irx3 expression in the ventral-most part of the hindbrain.

Taken together, these data establish that oligodendrocytes in the ventral spinal cord and hindbrain are generated from distinct ventral progenitor domains. Although the specification of ventral oligodendrocytes at different axial levels shows a similarity with respect to their dependence on Shh signaling (Alberta et al., 2001; Lu et al., 2002) and requirement for Olig1/2, our analysis of *Nkx6* mutant mice reveals crucial differences in the intrinsic programs that control Olig1/2 expression in the ventral spinal cord and hindbrain (Figures 6P and 6R). Considering that the loss of Nkx6 function has no significant influence on oligodendrocytes specified in dorsal positions of the spinal cord and hindbrain, it is apparent that also the upstream control of Olig1/2 expression in dorsal progenitors must be differently regulated as compared to their ventral counterparts.

Experimental Procedures

Mouse Mutants

The generation and genotyping of Nkx6.1- and Nkx6.2-deficient mice have previously been reported (Sander et al., 2000; Vallstedt et al., 2001).

Neural Tube Explant Cultures

Rhombomeres 4–6 from hindbrains of E10.5 mouse embryos (CB57) were divided into ventral, intermediate, and dorsal portions. Explants were embedded in collagen (Cohesion Technologies) and cultured as previously described (Sussman et al., 2000), with the exception that 1% FBS was replaced with 1% KCR (Invitrogen). Spinal cords from E10.5 and E12.5 mouse embryos (CB57) were divided into ventral and dorsal portions and cultured under same conditions. For assessments of FGF effects, 20 ng/ml FGF2 (Invitrogen) was added to media. Functional blocking of FGF signaling was performed by adding 25 μM SU5402 (Calbiochem) to the media. For assessments of BMP effects, 1 ng/ml BMP7 (R&D Systems) was added; blocking of BMP signaling was performed by adding 1 μg/ml Chordin and 1 μg/ml Noggin (R&D Systems).

Immunohistochemistry and In Situ Hybridization Histochemistry

Immunohistochemical localization of proteins was performed as described (Briscoe et al., 2000). Antibodies used were as follows: rabbit (r), mouse (m), and guinea pig (gp) Olig2 (Novitch et al., 2001), rat PDGFRα (PharMingen), m O4, rat MBP, r NG2 (Chemicon), m Pax7, m HB9, m Shh (DSHB), r Gsh1/2 (kind gift from Martin Goulding), m NeuN (Chemicon), r Nkx6.1, r Nkx2.2 (Briscoe et al., 2000). In situ hybridization histochemistry on sections or as whole mounts was performed (Schaeren-Wiemers and Gerfin-Moser, 1993) using mouse *Olig1*, *Olig2*, *Sox10*, *Dbx2*, *Nkx2.2*, *Pax3*, *Gsh1*, and *PDGFRα* probes.

Acknowledgments

We thank T. Jessell, B. Novitch, and M. Goulding for providing Olig2 and Gsh1/2 antibodies; and W. Richardson for PDGFRα cDNA. We are also grateful to T. Jessell and S. Applequist for comments on the manuscript. A.V. has received support from Boehringer Ingelheim Fonds. J.E. is supported by the Royal Swedish Academy of Sciences by a donation from the Wallenberg Foundation, the Swedish Foundation for Strategic Research, the Swedish National Research Council, the Wallenberg Foundation, the KI and the EC network grants Brainstem Genetics QLRT-2000-01467 and Stembridge QLG3-CT-2002-01141.

Received: August 4, 2004
Revised: October 22, 2004
Accepted: November 22, 2004
Published: January 5, 2005

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Neuron, Vol. 45, 41–53, January 6, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2004.12.028

Generation of Oligodendrocyte Precursor Cells from Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling

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Summary

In the developing spinal cord, early progenitor cells of the oligodendrocyte lineage are induced in the motor neuron progenitor (pMN) domain of the ventral neuroepithelium by the ventral midline signal *Sonic hedgehog* (*Shh*). The ventral generation of oligodendrocytes requires *Nkx6*-regulated expression of the bHLH gene *Olig2* in this domain. In the absence of *Nkx6* genes or *Shh* signaling, the initial expression of *Olig2* in the pMN domain is completely abolished. In this study, we provide the in vivo evidence for a late phase of *Olig* gene expression independent of *Nkx6* and *Shh* gene activities and reveal a brief second wave of oligodendrogenesis in the dorsal spinal cord. In addition, we provide genetic evidence that oligodendrogenesis can occur in the absence of hedgehog receptor *Smoothened*, which is essential for all hedgehog signaling.

Introduction

The spinal cord has served as an excellent model for studying the origin and molecular specification of oligodendrocytes in the developing central nervous system (CNS). Although oligodendrocytes are widely distributed in the adult spinal cord, recent findings have indicated that early progenitors of the oligodendrocyte lineage are induced from specific loci of the ventral neuroepithelium by the ventral midline signal *Sonic hedgehog* (*Shh*) (for reviews, see Richardson et al., 2000; Spassky et al., 2000; Miller, 2002). Under the influence of *Shh* morphogen, a number of transcription factors are selectively repressed (class I) or induced (class II) in the ventral neural progenitors (Briscoe et al., 2000), with each transcription factor having a different threshold response to the graded *Shh* signaling. As a result, these progenitor transcription factors display a nested pattern of expression along the dorsal-ventral axis. Based on their differential expression in the ventral spinal cord, the ventral neuroepithelium can be divided into five distinct domains (p0–p3 and motor neuron progenitor [pMN]), with each domain expressing a unique combination of progenitor genes and producing a specific neuronal cell type followed by either astrogliogenesis or oligodendrogenesis (Jessell, 2000; Zhou and Anderson, 2002). The pMN domain, which expresses *Nkx6* homeodomain transcription factors (Qiu et al., 1998; Briscoe et al., 2000; Vallstedt et al., 2001) and *Olig* bHLH transcription factors (Mizuguchi et al., 2001; Novitch et al., 2001), first produces motor neurons followed by oligodendrocyte precursor cells (OPCs) (Richardson et al., 1997; Sun et al., 1998; Fu et al., 2002) that subsequently migrate throughout the spinal cord before differentiating into myelinating oligodendrocytes. The sequential generation of motor neurons and OPCs from the pMN domain requires the expression of *Olig1* and *Olig2* transcription factors in this domain, and disruption of the *Olig* genes leads to the loss of both motor neurons and oligodendrocytes in the spinal cord (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Based on these and other observations, it is believed that, in the spinal cord, early OPCs originate from the pMN domain, and oligodendrocyte development is coupled to motor neuron development (Zhou et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002).

The possible contribution of dorsal neuroepithelium to oligodendrocyte development in the spinal cord has been under intensive investigation and considerable debate. In the developing chick embryos, some early transplantation studies suggested that oligodendrocytes were generated from both dorsal and ventral spinal cord (Cameron-Curry and Le Douarin, 1995). However, similar chick-quail grafting experiments argued that dorsal spinal neuroepithelial cells only produced astrocytes but not oligodendrocytes (Pringle et al., 1998). Recent studies in rodents suggested that glial-restricted progenitor (GRP) cells, which can give rise in vitro to OPCs and astrocytes, could be derived from both dorsal and ventral spinal cords (Rao et al., 1998; Gregori et al., 2002). Moreover, in vitro culture of dorsal mouse spinal cord explants, like that of its ventral counterpart, can also produce OPCs, although with a significant delay. In the mean time, the intermediate region located between the dorsal and ventral explants failed to generate OPCs in culture (Sussman et al., 2000), arguing against the possibility of dorsal invasion of OPCs from the ventral region. Together, these experiments indicated that the dorsal spinal neuroepithelial cells in mammals have an intrinsic and independent potential to produce oligodendrocytes under appropriate conditions. However, it is not known whether this potential is realized during the in vivo development of mouse spinal cord, as it has been argued that, in culture, neural progenitor cells may lose their positional cues and behave differently from in vivo in response to exogenous factors (Gabay et al., 2003; Stiles, 2003). For instance, bFGF can ventralize dorsal neural progenitor cells in vitro, resulting in an arbitrary induction *Olig2* expression and oligodendrocyte differentiation (Gabay et al., 2003; Chandran et al., 2003; Kessaris et al., 2004).

To investigate whether OPCs can be derived from the dorsal spinal cord in vivo, we examined oligodendrocyte development in *Nkx6.1*$^{-/-}$ *Nkx6.2*$^{-/-}$ and *Shh*$^{-/-}$ mutant

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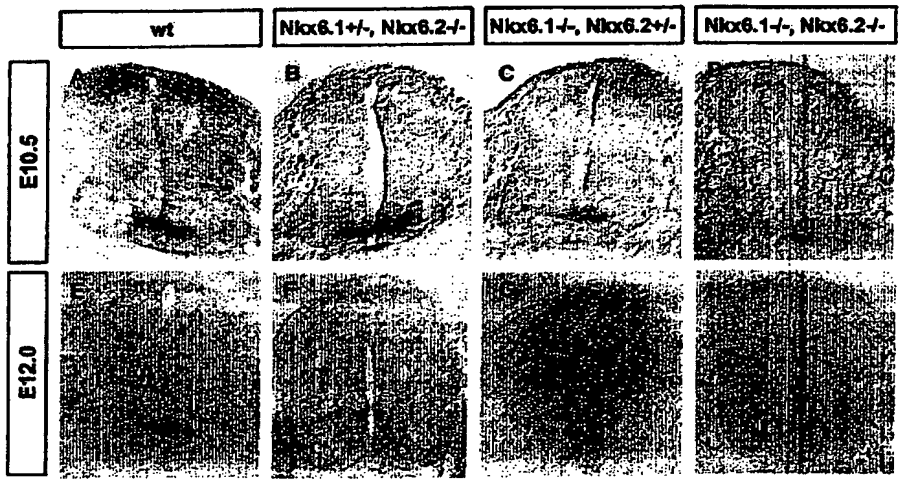

Figure 1. Early Expression of *Olig2* in Various *Nkx6* Mutant Spinal Cords

Transverse spinal cord sections from E10.5 (A–D) and E12.0 (E–H) embryos of various *Nkx* genotypes were subjected to in situ hybridization with *Olig2* riboprobe. The *Olig2* expression in the pMN domain was regulated by the redundant activities of *Nkx6.1* and *Nkx6.2* and was completely suppressed in *Nkx6*$^{-/-}$ double mutants.

spinal cords, in which the early ventral oligodendrogenesis from the pMN domain is abolished, so that the potential dorsal oligodendrogenesis could be unmasked. Our studies on oligodendrogenesis in *Nkx6*$^{-/-}$ double mutants and *Shh*$^{-/-}$ mutants uncovered a transient production of OPCs in the dorsal spinal cord. The dorsal generation of OPCs was also observed in wild-type spinal cords and was confirmed by in vitro culture of dorsal spinal cord explants. Together, these observations suggest an *Nkx*- and *Shh*-independent mechanism for *Olig* gene expression in the dorsal spinal cord after neurogenesis and provide evidence for a late phase of oligodendrogenesis independent of motor neuron development in the dorsal spinal cord.

Results

Nkx6 Dosage-Dependent *Olig* Gene Expression in the Ventral Ventricular Zone during Neurogenesis

Previous studies have demonstrated that *Nkx6.1* and *Nkx6.2* have redundant functions in controlling motor neuron specification, with *Nkx6.1* having a larger effect than *Nkx6.2* (Vallstedt et al., 2001). To examine the effects of different levels of *Nkx6* gene activity on oligodendrocyte development in embryonic spinal cord, we first examined the early expression of *Olig2*, the principal *Olig* gene responsible for motor neuron and oligodendrocyte development (Lu et al., 2002; Takebayashi et al., 2002), in the ventral spinal cords of various *Nkx6* mutants prior to oligodendrogenesis stages. Consistent with the previous findings (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000), at E10.5 and E12.0, *Olig 2* was exclusively expressed in the pMN domain of the wild-type spinal cord (Figures 1A and 1E). In heterozygous embryos (*Nkx6.1*$^{+/-}$, *Nkx6.2*$^{+/-}$, or *Nkx6.1*$^{+/-}$ *Nkx6.2*$^{+/-}$) and *Nkx6.2*$^{-/-}$ homozygous embryos, *Olig2* expression was not significantly affected (data not shown). In *Nkx6.1*$^{+/-}$ *Nkx6.2*$^{-/-}$ embryos, expression of *Olig2* in the ventral ventricular zone was slightly decreased (Figures 1B and 1F). However, *Olig2* expression was markedly reduced in *Nkx6.1*$^{-/-}$ (Liu et al., 2003) or *Nkx6.1*$^{-/-}$ *Nkx6.2*$^{+/-}$ embryos (Figures 1C and 1G) and completely eliminated in *Nkx6.1*$^{-/-}$ *Nkx6.2*$^{-/-}$ (referred to as *Nkx6*$^{-/-}$ hereafter) compound mutants (Figures 1D and 1H). Collectively, these results indicated a dosage-dependent regulation of *Olig2* expression in the ventral spinal cord by *Nkx6* transcription factors, and its expression is largely dependent on *Nkx6.1* activity but to a lesser extent on *Nkx6.2* activity.

Delayed and Dorsal Expression of *Olig* Genes in *Nkx6*$^{-/-}$ Spinal Cords during Gliogenesis

To investigate whether the lack of *Olig* expression in the pMN domain leads to a complete inhibition of oligodendrogenesis in the spinal cord, we examined OPC generation and differentiation at progressively later stages of embryonic development in *Nkx6*$^{-/-}$ double mutants. At E13.5, many *Olig1*+ and *Olig2*+ OPCs had already migrated out of the ventral ventricular zone in wild-type spinal cords (Figures 2A and 2C). As expected, no migratory *Olig*+ cells were observed outside the ventricular zone in *Nkx6*$^{-/-}$ mutants. Surprisingly, a small number of *Olig1*+ and *Olig2*+ cells were detected in the mutants in both dorsal and ventral ventricular zone (Figures 2B and 2D). The ventral expression of *Olig* genes in *Nkx6*$^{-/-}$ mutants occurred at approximately the same position as the pMN domain. These data suggested a *Nkx6*-independent regulation of *Olig* gene expression in both the dorsal and ventral spinal cord during oligodendrogenesis stages. The dorsal expression of *Olig* genes in the mutants became more apparent at E14.5, when a small number of *Olig1*+ and *Olig2*+ OPCs started to

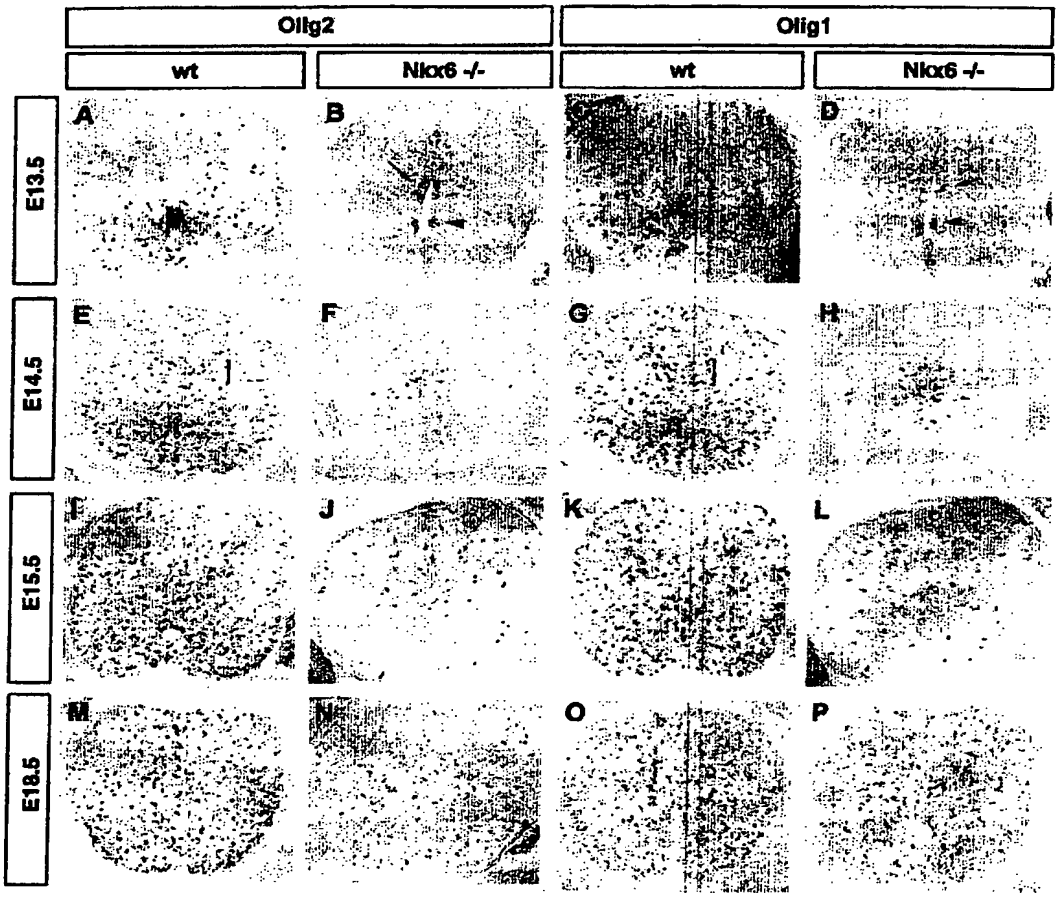

Figure 2. Late and Dorsal Expression of *Olig2* Gene in *Nkx6*$^{-/-}$ Spinal Cords at the Thoracic Level

Transverse sections from E13.5 (A–D), E14.5 (E–H), E15.5 (I–L), and E18.5 (M–P) spinal cords of wild-type and *Nkx6*$^{-/-}$ embryos were subjected to in situ hybridization with riboprobes for *Olig2* and *Olig1*. At E13.5, *Olig1* and *Olig2* expression was upregulated in *Nkx6*$^{-/-}$ double mutants in both dorsal (indicated by arrows) and ventral (indicated by the arrowheads) positions. At E14.5 and later stages, *Olig1*+ and *Olig2*+ cells migrated into the surrounding regions in a dorsal to ventral gradient, in contrast to that seen in the wild-type spinal cords. The positions of dorsal-derived *Olig*+ cells in E14.5 wild-type spinal cord are outlined by a square bracket in (E) and (G).

migrate away from the dorsal ventricular zone (Figures 2F and 2H). Interestingly, very few migratory OPCs were produced from the *Olig*-expressing ventral ventricular zone of the mutant spinal cord. Thus, a vast majority of *Olig*+ OPC cells in *Nkx6*$^{-/-}$ mutants appeared to originate from the dorsal ventricular zone. At this stage, a distinct population of *Olig1*+ and *Olig2*+ cells were also closely associated with the dorsal ventricular or subventricular zone of the wild-type spinal cords (Figures 2E and 2G), and there was an apparent discontinuity between this group of *Olig*+ cells and the ventral-derived *Olig*+ cells (more apparent in Figures 5A, 5I, 8C, and 8E). Together, these observations suggest that a small number of *Olig*+ OPCs are produced from the dorsal neuroepithelial cells in both normal and *Nkx6*$^{-/-}$ spinal cords.

At E15.5, the number of *Olig1/2*+ cells in *Nkx6*$^{-/-}$ mutants was further increased, but most of them remained confined to the dorsal spinal cords (Figures 2J and 2L). In contrast, a higher percentage (55%) of *Olig1/2*+ OPCs were found in the ventral half of wild-type spinal cord (Figures 2I and 2K), and they were presumably derived from the ventral neuroepithelial cells. By E18.5, *Olig1/2*+ cells were distributed more or less evenly throughout the entire spinal cord in *Nkx6*$^{-/-}$ mutants (Figures 2N and 2P), suggesting that the dorsal-derived *Olig*+ OPCs in *Nkx6*$^{-/-}$ mutants migrated progressively from the dorsal to the ventral spinal cord. However, the number of *Olig*+ cells remained significantly smaller than that in wild-type embryos (Figures 2M and 2O; Figure 3Q).

Delayed Appearance of Other Oligodendrocyte Markers in *Nkx6*$^{-/-}$ Mutants

One critical issue for the dorsal-derived *Olig*+ cells is whether they are capable of differentiating further along the oligodendrocyte lineage. To address this question,

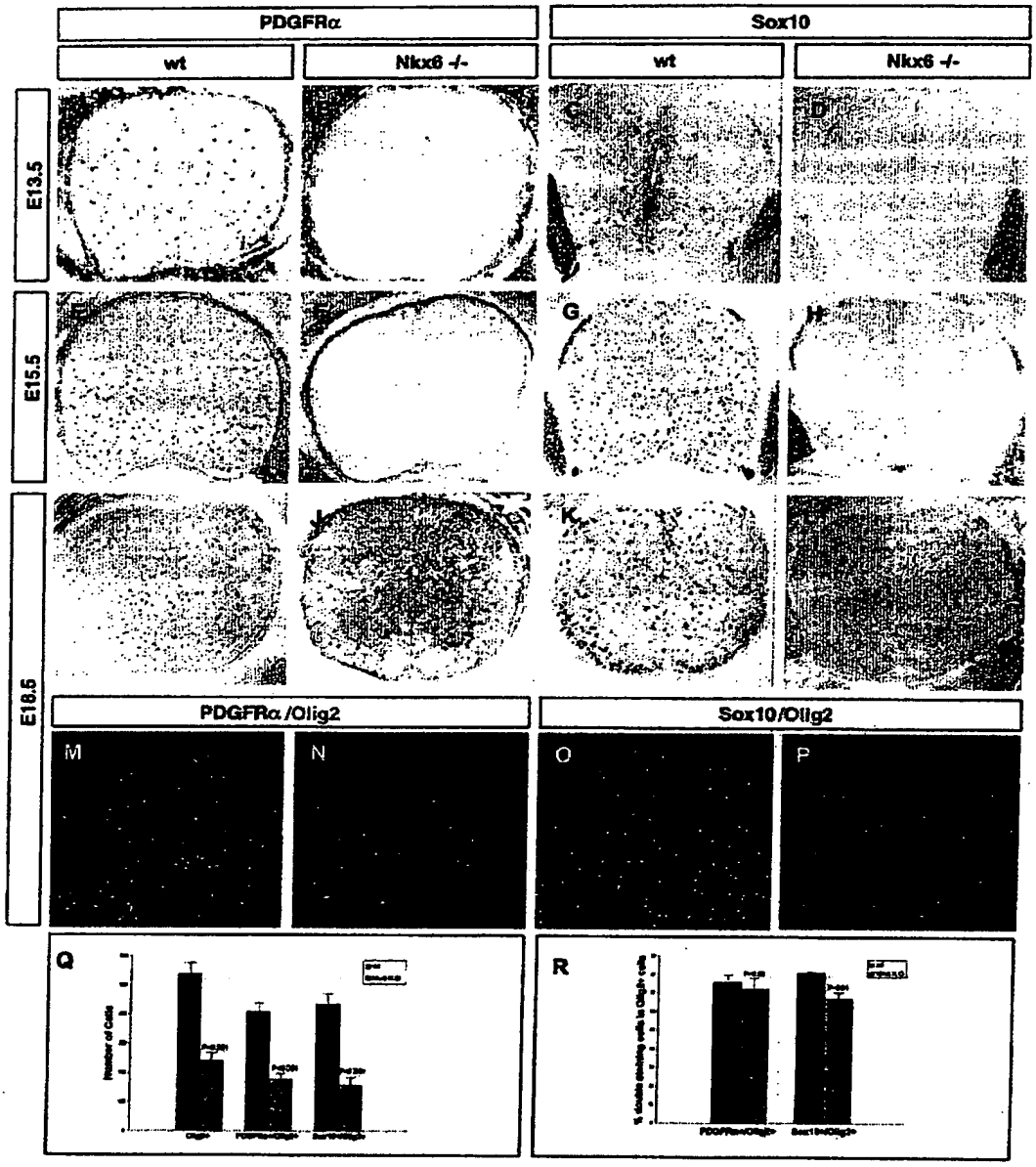

Figure 3. Delayed and Reduced Production of *PDGFRα+* and *Sox10+* OPCs in *Nkx6*$^{-/-}$ Mutant Spinal Cords

(A–L) Spinal cord sections from E13.5 (A–D), E15.5 (E–H), and E18.5 (I–L) wild-type and mutant embryos were subjected to in situ hybridization with *PDGFRα* or *Sox10* riboprobes. A smaller number of *PDGFRα+* and *Sox10+* cells started to emerge in E18.5 *Nkx6*$^{-/-}$ spinal cords.

(M–P) Double immunostaining of E18.5 wild-type and mutant spinal cord with *Olig2* (green) and *PDGFRα* ([M] and [N], red) or *Sox10* ([O] and [P], red).

(Q) The number of Olig2+ single-positive OPCs and *Olig2+/PDGFRα+* or *Olig2+/Sox10+* double-positive cells per spinal cord section in E18.5 wild-type or *Nkx6*$^{-/-}$ mutants (average of three sections).

(R) The percentage of *Olig2+* cells that coexpress *PDGFRα* or *Sox10* in E18.5 wild-type or *Nkx6*$^{-/-}$ mutants. Statistical analyses in (Q) and (R) were performed with Student's t test.

we examined the expression of several oligodendrocytic markers (e.g., *PDGFRα*, *Sox10*, and *MBP*) downstream of *Olig1/2* in *Nkx6*$^{-/-}$ spinal cords. In wild-type spinal cords, expression of *PDGFRα* and *Sox10* is restricted to oligodendrocyte lineage (Pringle et al., 1992; Stolt et al., 2002) and can be detected in the ventral spinal cord at E13.5, whereas their expression in *Nkx6*$^{-/-}$ mutants was not observed until E18.5 (Figures 3A–3L), indicating

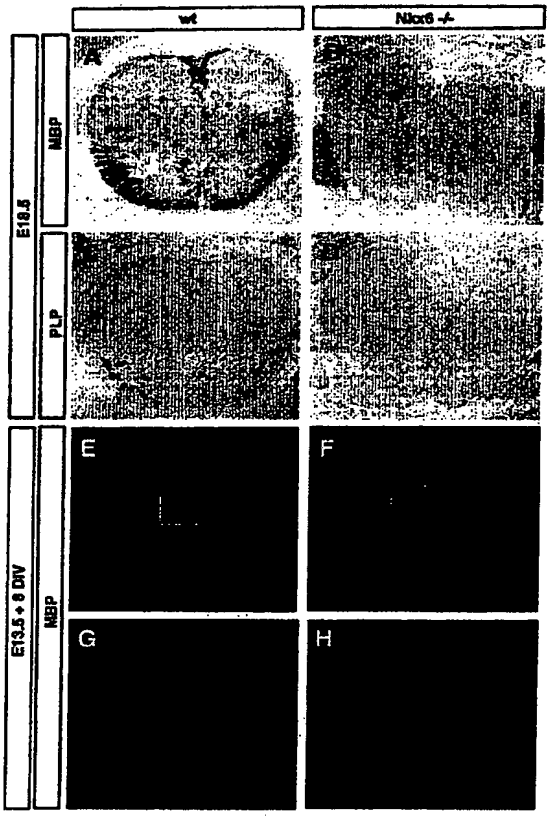

Figure 4. Disrupted Expression of *MBP* and *PLP* in *Nkx6*$^{-/-}$ Mutant Spinal Cords

(A–D) Spinal cord sections were prepared from E18.5 wild-type (A and C) and mutant (B and D) animals and hybridized with *MBP* (A and B) and *PLP* (C and D) riboprobes.

(E–H) Spinal cord explants isolated from E13.5 wild-type and *Nkx6*$^{-/-}$ embryos were cultured on floating membranes for 8 days before they were subjected to anti-*MBP* whole-mount immunostaining. (G) and (H) are higher magnifications of (E) and (F), respectively, showing *MBP*+ myelinating axons.

a significant delay of OPC differentiation. Double immunostaining at this stage confirmed that a high percentage of *Olig2*+ cells in *Nkx6* mutants coexpressed *PDGFRα* and *Sox10* (Figures 3M–3P and 3R), although the total number of *Olig2*+/*PDGFRα*+ and *Olig2*+/*Sox10*+ cells per spinal cord section remained significantly smaller (Figure 3Q). Similarly, expression of the mature oligodendrocyte markers *MBP* and *PLP* in mutant spinal cords was also affected. In normal embryos, many *MBP*+/*PLP*+ oligodendrocytes were seen in the ventral spinal cord at E18.5 (Figures 4A and 4C). However, no *MBP*+/*PLP*+ cells were detected in *Nkx6*$^{-/-}$ mutants at this stage (Figures 4B and 4D). Together, these results suggest that the dorsal-derived *Olig*+ cells in *Nkx6*$^{-/-}$ spinal cords can progress along the oligodendrocyte lineage, but they develop and mature much more slowly than the early-born ventral OPCs.

To assess whether dorsal-derived OPCs in *Nkx6*$^{-/-}$ mutants are capable of differentiating into mature oligodendrocytes in vitro, we isolated spinal cord explants from E13.5 wild-type and mutant embryos and cultured them on floating membranes. Following 8 days of culture, a small number of *MBP*+ cells started to emerge in mutant tissues (Figures 4E and 4F). Moreover, *MBP*+ fibers, indicators of myelinating axons, were observed in the axon-enriched medial (ventral) regions of both normal and mutant explants (Figures 4G and 4H). These results suggest that the dorsal-derived OPCs in *Nkx6*$^{-/-}$ mutants are capable of differentiating into *MBP*+ mature oligodendrocytes and form myelin sheaths, at least in vitro.

Olig+ OPCs Are Briefly Produced from the Dorsal Neuroepithelial Cells and Transiently Coexpress Some Dorsal Neural Progenitor Markers

To verify the dorsal origin of a subset of *Olig*+ OPCs in both normal and *Nkx6*$^{-/-}$ spinal cords, sections from E14.5 embryos were subjected to double immunostaining with antibodies against *Olig2* and two dorsal neural progenitor markers, *Pax7* and *Mash1*. During neurogenesis and early gliogenesis, *Pax7* is expressed in the entire dorsal ventricular zone of the spinal cord (Goulding et al., 1993), whereas *Mash1* expression in the dorsal spinal cord is restricted to the dorsal interneuron progenitor domains dI3–dI5 (Gross et al., 2002; Muller et al., 2002; Caspary and Anderson, 2003). Double immunostaining revealed that a subpopulation of *Olig2*+ cells was closely associated with the *Pax7*+ and *Mash1*+ dorsal neuroepithelial cells in both genotypes (Figures 5A–5J). Moreover, a small number of migratory *Olig2*+ cells in the dorsal ventricular zone or immediately adjacent regions coexpressed *Pax7* and *Mash1* (arrows in Figures 5B–5D and 5F–5H; insets in Figures 5I and 5J). These colabeling data strongly suggested that some *Olig2*+ cells arose from the dI3–dI5 domains of dorsal neural progenitor cells in both normal and *Nkx6*$^{-/-}$ spinal cords and that the dorsal-derived *Olig2*+ cells retained the expression of dorsal markers *Pax7* and *Mash1* for a brief period of time. Although only about 8% of *Olig2*+ cells were also *Pax7*+ in E14.5 wild-type spinal cord, this may be an underestimate of the percentage of dorsal-derived OPC population due to the rapid downregulation of *Pax7* after they migrate away into the surrounding region. Intriguingly, the total number of *Olig2*+/*Pax7*+ and *Olig2*+/*Mash1*+ cells in *Nkx6*$^{-/-}$ double mutants was significantly larger than that in normal embryos (Figure 5M). One plausible explanation is that the dorsal-derived *Olig2*+ cells in mutant spinal cords proliferated more rapidly, possibly due to the lack of competition from the ventral-derived OPCs for mitogens. Alternatively, expression of *Pax7* and *Mash1* in dorsal-derived OPCs may be downregulated more slowly in *Nkx6* mutants.

To confirm our mapping of the origin of dorsal OPCs, we also compared the expression of *Olig2* with that of two other neural progenitor genes, *Dbx1* and *Dbx2*. Previous studies have shown that *Dbx1* is expressed in the dorsal-ventral boundary of the embryonic spinal cord, whereas *Dbx2* is expressed in the dI6 domain of the dorsal spinal cord and the p0 and p1 domains of the ventral spinal cord (Briscoe et al., 2000; Caspary

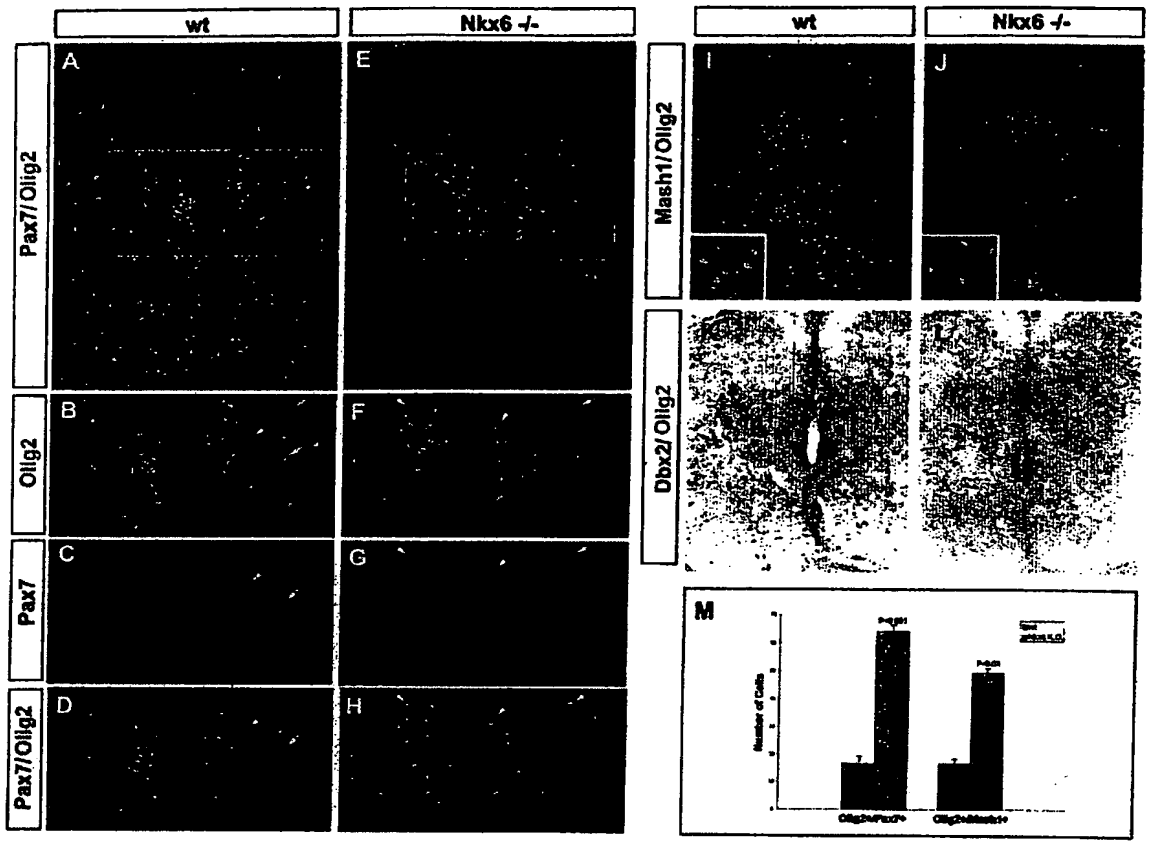

Figure 5. *Olig*+ Cells Originated from *Pax7*+ and *Mash1*+ but *Dbx*− Dorsal Interneuron Progenitor Domains

(A–H) Coexpression of *Pax7* and *Olig2* in the dorsal spinal cord. E14.5 spinal cord sections from wild-type (A–D) and *Nkx6*$^{-/-}$ (E–H) embryos were simultaneously immunostained with antibodies against *Olig2* (in green) and *Pax7* (in red). (B)–(D) and (F)–(H) are the higher magnifications of the boxed areas in (A) and (E), respectively. In both genotypes, a group of *Olig2*+ cells were produced from the *Pax7*+ dorsal ventricular zone, and some of the *Olig2*+ cells retained the expression of *Pax7* (represented by arrows in [B]–[D] and [F]–[H]).

(I–L) E14.5 wild-type and *Nkx6*$^{-/-}$ spinal cord sections were double immunostained with anti-*Olig2* and anti-*Mash1* antibodies (I and J), or subject to in situ hybridization with *Dbx2* riboprobe followed by anti-*Olig2* immunohistochemistry (K and L). The *Olig2*+/*Mash1*+ double-positive cells are represented in insets in (I) and (J). The dorsal-derived *Olig2*+ cells in (K) and (L) are outlined by a square bracket.

(M) Statistical analyses (Student's t test) of *Olig2*+/*Pax7*+ and *Olig2*+/*Mash1*+ double-positive cells in wild-type and *Nkx6*$^{-/-}$ mutants per section.

and Anderson, 2003). Double labeling of E14.5 spinal cord sections demonstrated that the dorsal *Olig2*+ cells lay immediately dorsal to *Dbx2* expression (Figures 5K and 5L) but well above *Dbx1* expression (data not shown), indicating that the dorsal *Olig2*+ cells were derived from regions above the dI6 domain. This result is consistent with the idea that dorsal *Olig2*+ cells are primarily derived from the *Mash1*+ dI3–dI5 dorsal interneuron progenitor cells.

To further confirm that the dorsal spinal cord has an independent potential to generate OPCs, we dissected the dorsal and ventral halves of spinal cord from E11.5 mouse embryos and cultured them separately in collagen gel or on floating membranes in the absence of exogenous bFGF, which is known to induce *Olig2* expression in cultured dorsal neural progenitor cells (Gabay et al., 2003; Chandran et al., 2003). In E11.5 mouse spinal cord, OPCs were not produced from the pMN domain yet (Figures 6A and 6B), excluding the possibility of dorsal invasion of ventral OPCs. Following 3 days of in vitro culture (equivalent to E14.5), a small number of *Olig2*+ cells started to emerge from the dorsal explants and coexpressed Pax7 (Figure 6C). Expression of later OPC markers *PDGFRα* and NG2 in dorsal explants was seen after 4–6 days of culture, and that of mature markers GalC and *MBP* was seen after 6–8 days of culture in vitro (Figures 6E–6L). Together, these data indicated that the dorsal spinal cord explants have an intrinsic potential to produce OPCs, and the schedule of OPC generation and differentiation in dorsal explant culture

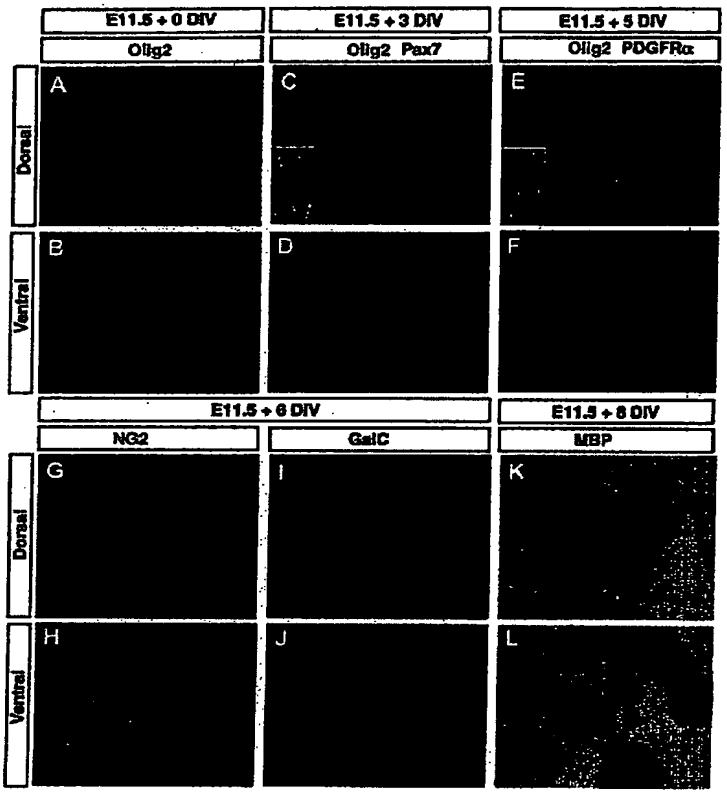

Figure 6. Generation of Oligodendrocytes from Dorsal Spinal Cord Explant Culture

Mouse E11.5 spinal cord tissues isolated from the thoracic level were bisected into dorsal and ventral halves and cultured separately in collagen gel (A–J) or on floating membrane (K and L) for various days in vitro (DIV) as indicated. Explants were then subject to immunofluorescent staining with antibodies or in situ hybridization with *MBP*.

is similar to that in vivo. In agreement with previous findings (Gabay et al., 2003; Chandran et al., 2003), addition of exogenous bFGF dramatically increased the number of *Olig2*+ OPCs in both dorsal and ventral explants (data not shown).

Generation of Dorsal OPCs in the Absence of *Shh* Signaling

The generation of OPCs in the dorsal spinal cord suggests a *Shh*-independent pathway for oligodendrogenesis, since cell fate specification in the dorsal spinal cord is primarily regulated by dorsal midline signals, notably BMPs (Dickinson et al., 1995; Liem et al., 1995). To test the possibility, we examined whether OPCs' production from dorsal explants can be blocked by anti-*Shh* antibody. In contrast to the previous finding that *Shh* was partially required for O4 expression in dorsal explants (Sussman et al., 2000), we found that anti-*Shh* antibody had no apparent effect on *Olig2* gene expression in dorsal explants, although it dramatically inhibited *Olig2* expression in the ventral explants (Figures 7A–7D). To provide genetic evidence that oligodendrogenesis can occur independent of hedgehog signaling, we differentiated ES (embryonic stem) cells deficient in pan-hedgehog signaling component *Smoothened* (*Smo*$^{-/-}$; Wijgerde et al., 2002) in the presence of retinoid acid and found that GalC+ oligodendrocytes were formed from both normal and *Smo*$^{-/-}$ mutant ES cells at a comparable efficiency (Figures 7E and 7F).

We next examined oligodendrocyte development in *Shh*$^{-/-}$ mutant spinal cord to confirm that *Shh* signaling is not responsible for dorsal oligodendrogenesis in vivo. In *Shh*$^{-/-}$ mutants, the spinal cord is dorsalized, and most of the ventral structures including the pMN domains are missing (Chiang et al., 1996; Pierani et al., 1999). Consistent with some earlier findings that *Shh* is required for ventral oligodendrogenesis (Lu et al., 2000; Alberta et al., 2001), no early *Olig1/2*+ OPCs were produced in *Shh* mutant spinal cords at or before E13.5 (Figures 8A and 8B; data not shown). However, at E14.5, a small number of *Olig1*+ and *Olig2*+ cells started to appear in the dorsal region of the mutant spinal cords (Figures 8D and 8F). By E18.5, a larger number of *Olig2*+ cells were observed throughout the mutant spinal cord (Figure 8H).

Similar to our data in *Nkx6*$^{-/-}$ mutants, oligodendrocyte lineage progression in *Shh* null spinal cord was also delayed. Expression of *PDGFR* and *Sox10* was not detected until E18.5 (Figures 8I–8L), and no *MBP* expression was observed at perinatal stages (Figures 8M and 8N). However, when spinal cord explants isolated from E18.5 mutant embryos were cultured in vitro for 2 additional days, a small number of *MBP*+ cells started to emerge in mutant tissues (Figures 8O and 8P), indicating

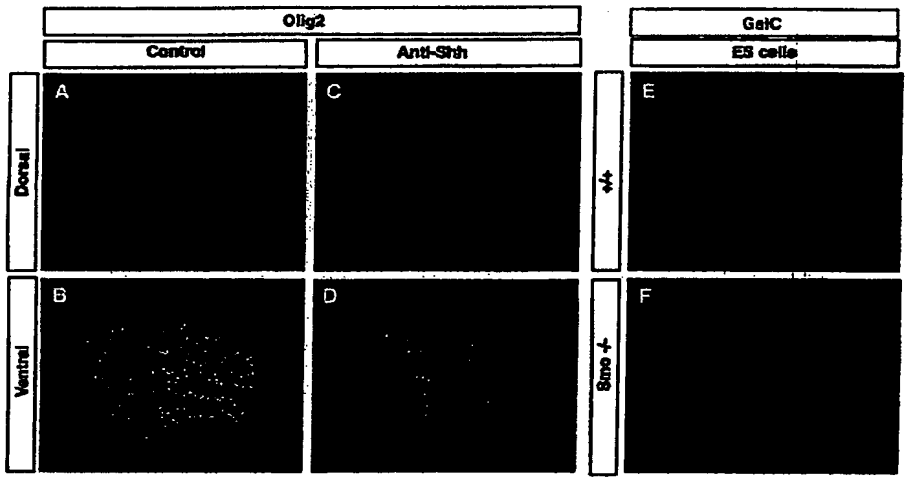

Figure 7. *Shh*-Independent Generation of Oligodendrocytes in Spinal Explants and ES Cells

(A–D) Inhibition of *Olig* gene expression by anti-*Shh* antibody in ventral explants but not in dorsal explants. Dorsal and ventral spinal cord explants from E11.5 wild-type embryos were cultured in collagen gel for 3 days in the absence (A and B) or presence (C and D) of anti-*Shh* antibody prior to immunostaining with anti-*Olig2*.

(E and F) Differentiation of wild-type (E) and *Smo*$^{-/-}$ (F) ES cells into GalC+ oligodendrocytes.

that the dorsal-derived OPCs in *Shh* mutants are able to differentiate into *MBP*+ mature oligodendrocytes as well.

Discussion

This study reveals an *Nkx6*- and *Shh*-independent mechanism for a late phase of *Olig* gene expression in the dorsal spinal cord after the onset of early oligodendrogenesis from the pMN domain (Figure 9). The late *Olig* gene expression is associated with a brief wave of oligodendrogenesis in the dorsal spinal cord. These findings provide evidence for multiple origins of OPC generation involving distinct inductive signals during spinal cord development.

Nkx6-Independent Mechanisms for *Olig* Gene Expression and Oligodendrogenesis in the Spinal Cord

Previous work had demonstrated that *Nkx6.1* and *Nkx6.2* have redundant functions in controlling motor neuron specification in the spinal cord (Vallstedt et al., 2001). Consistent with this line of study, our findings indicate that *Nkx6.1* and *Nkx6.2* have redundant activities in regulating the early expression of *Olig2* in the pMN domain, with *Nkx6.1* exerting a larger effect than *Nkx6.2* (Figure 1). In the absence of both *Nkx6.1* and *Nkx6.2*, the initial expression of *Olig 2* in the pMN domain is completely abolished. In keeping with the idea that early progenitors of the oligodendrocyte lineage are derived from the *Olig2*+ pMN domain of the ventral spinal cord, the loss of *Olig2* expression in the pMN domain in *Nkx6*$^{-/-}$ mutants was associated with the failure of production of early OPC cells from the ventral spinal cord (Figure 2).

Despite the lack of early expression of *Olig2* in the pMN domain in *Nkx6*$^{-/-}$ mutants, a low level of *Olig1* and *Olig2* expression started to be detected in both the ventral and dorsal ventricular zone after the onset of oligodendrogenesis (Figure 2). In the dorsal spinal cord, *Olig* gene expression was detected in *Pax7*+/*Mash1*+ dI3–dI5 dorsal neural progenitor domains starting at E13.5 (Figures 2 and 5). In the ventral spinal cord, a small number of ventral ventricular cells started to express *Olig1* and *Olig2* genes at approximately the same position as the pMN domain (Figures 2B and 2D). Together, these results indicate an *Nkx6*-independent regulation of *Olig* gene expression in both the dorsal and ventral spinal cord. The late phase of *Olig* gene expression in the dorsal spinal neuroepithelium was also observed in wild-type spinal cord, mostly at E14.5 (Figures 5 and 8) but occasionally at E13.5 (data not shown). Thus, the dorsal ventricular *Olig2* expression appeared to be slightly advanced or enhanced in *Nkx6* mutants. This enhancement might partially account for the increased population of *Olig2*+/*Pax7*+ cells in the mutants. Interestingly, no *Olig* expression was observed in E13.5 *Shh* mutants (Figure 8), suggesting that the premature or enhanced dorsal ventricular *Olig* expression was not simply due to the loss of ventral patterning, but more specifically associated with the absence of *Nkx6* gene expression.

The dorsal *Olig* gene expression was associated with a transient production of OPCs starting at around E14.5, about 2 days later than the ventral oligodendrogenesis from the pMN domain (Figure 9B). In both wild-type and *Nkx6*$^{-/-}$ spinal cords, migratory *Olig1/2*+ OPCs were briefly produced from the dorsal neural progenitor cells. Several lines of evidence strongly suggest that these OPC cells are generated de novo from the dorsal ventricular cells, instead of having migrated up from the ventral cord. First, many dorsal *Olig2*+ cells coexpressed sev-

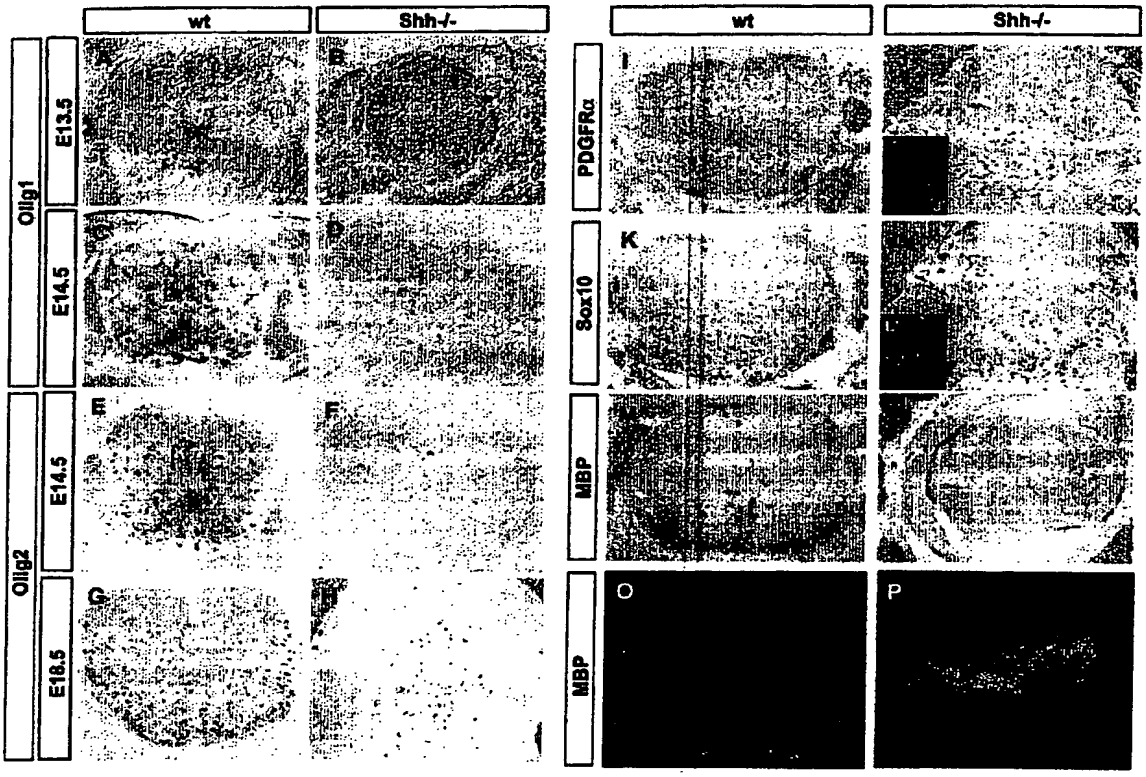

Figure 8. Oligodendrocyte Development in *Shh* Mutant Spinal Cord

(A–H) Dorsal generation of *Olig1/2*+ OPC cells in *Shh* mutant spinal cords. Spinal cord sections from E13.5 (A and B), E14.5 (C–F), and E18.5 (G and H) wild-type (A, C, E, and G) or *Shh*$^{-/-}$ (B, D, F, and H) embryos were subjected to in situ hybridization (ISH) with *Olig1* (A–D) and *Olig2* (E–H) riboprobes. At E13.5, *Olig*+ OPCs were generated from the ventral neuroepithelium in wild-type embryos but not in *Shh* mutants. At E14.5, a small group of *Olig1*+ and *Olig2*+ cells were associated with the dorsal neuroepithelium in both wild-type and *Shh* mutants. Dorsal *Olig*+ cells are outlined by a square bracket in (C) and (E).

(I–N) Distribution and differentiation of OPC cells in *Shh* mutant spinal cords. (I–N) Expression of *PDGFRα* (I and J), *Sox10* (K and L), and *MBP* (M and N) in E18.5 wild-type (I, K, and M) and *Shh* mutant (J, L, and N) spinal cords. *Olig2*+/*PDGFRα*+ and *Olig2*+/*Sox10*+ cells in mutants are represented in insets (J′) and (L′), respectively.

(O and P) Spinal cord tissues from E18.5 wild-type and *Shh* mutant embryos were isolated and cultured on polycarbonate membranes for 2 days in vitro and then subjected to whole-mount ISH with the *MBP* probe. A small number of *MBP*+ cells emerged in the mutant tissue.

eral dorsal neural progenitor genes such as *Pax7* and *Mash1* (Figure 5). Second, dorsal neural explants isolated from E11.5 spinal cord prior to ventral oligodendrogenesis can give rise to OPCs and oligodendrocytes on schedule as in vivo (Figure 6). Third, dorsal *Olig2* expression in *Nkx6* mutants was no later than its ventral expression. At E13.5 and E14.5, there was an apparent discontinuity of dorsal *Olig2*+ cells and ventral *Olig2*+ cells in the mutants (Figures 2B, 2D, 5E, and 5J), and the number of *Olig2*+ cells in E14.5 dorsal half far exceeded that of ventral *Olig2*+ cells, arguing against the dorsal migration of *Olig2*+ cells at least at these early stages. However, it is plausible that some ventral *Olig2*+ cells could migrate dorsally after E14.5 and contribute to the dorsal OPC population. Finally, OPCs can be produced from the dorsal region of *Shh* mutant spinal cord, which lacks the pMN domain (Pierani et al., 1999) and presumably the ventral oligodendrogenesis (Figure 8), although we can not absolutely exclude the possibility that a few *Olig*+ cells could also be generated from the remaining p0 and p1 domains in the most ventral region (Pierani et al., 1999). Together, these observations indicate that dorsal *Olig2*+ cells can be produced locally from the dorsal neuroepithelial cells in normal and *Nkx6*/*Shh* mutant spinal cords. Since the dorsal *Olig2*+/*Pax7*+/*Mash*+ OPCs were observed in the thoracic or even more caudal regions (data not shown), it is unlikely that they represent the longitudinally migrating cells from the rostral hindbrain.

Similar to the early ventral OPCs, the dorsal-derived *Olig*+ OPCs are capable of migration, proliferation, and differentiation along the oligodendrocytic lineage after they migrate out of the germinal zone. The delayed appearance of *PDGFRa*+ and *Sox10*+ OPCs in the *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$ mutants indicated that the late-born dorsal OPCs develop and differentiate much later than the early-born ventral oligodendrocytes. Although no *MBP* and *PLP* expression was observed in both mutants at

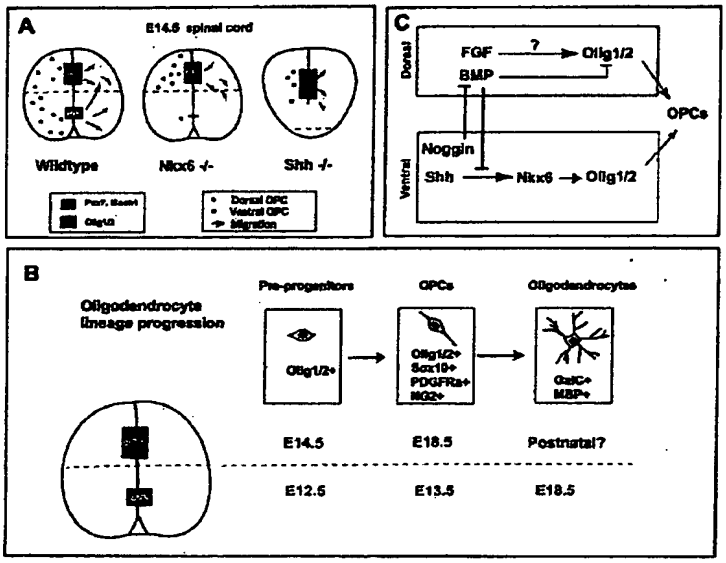

Figure 9. Proposed Origins and Molecular Specification of Oligodendrocytes in the Spinal Cord

(A) Proposed oligodendrocyte development in *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$ mutant spinal cords. In the wild-type, a vast majority of OPCs are derived from the ventral pMN domain. A subpopulation of OPCs is also generated from the dorsal dI3–dI5 domains independent of *Nkx6* and *Shh* activities. The arrows represent the possible migration directions.

(B) Time schedule of lineage progression for both dorsal- and ventral-derived OPCs. The generation of dorsal OPCs is about 2 days later than that of ventral OPCs. In general, there is a parallel delay in dorsal OPC generation and their differentiation.

(C) Proposed molecular pathways for dorsal and ventral oligodendrogenesis. *Shh* and *BMPs* are known to be the major inducer and repressor of oligodendrogenesis, respectively. In the ventral spinal cord, OPCs are generated in a *Shh/Nkx6*-dependent mechanism. Repression of *BMP* signaling by the notochord-derived Noggin may also contribute to ventral oligodendrogenesis. In the dorsal spinal cord, OPCs are generated independent of the *Shh/Nkx6* pathway and may result from a combination of FGFs and progressive loss of BMP inhibition over time.

E18.5, OPCs in both mutants could mature into *MBP*+ oligodendrocytes (Figures 4F and 8P) or even myelinate axons (Figure 4H) if they were allowed to develop further in vitro. Consistently, OPCs generated in the dorsal explants of normal embryos could also differentiate into mature oligodendrocytes (Figure 6). In general, there appears to be a parallel delay of OPC generation and their terminal differentiation as observed in other genetic mutants (Qi et al., 2003; Liu et al., 2003).

A *Shh*-Independent Pathway for Oligodendrogenesis in the Developing Spinal Cord

Early studies demonstrated that blockade of *Shh* signaling can inhibit oligodendrogenesis both in vivo and in vitro (Orentas et al., 1999; Davies and Miller, 2001; Tekki-Kessaris et al., 2001). Thus, it has been believed that *Shh* signaling is required for the development of oligodendrocytes in the entire CNS. However, the observations that OPCs can be produced from dorsal spinal cord explants in the presence of anti-*Shh* antibody (Figure 7) or from dissociated dorsal neural progenitor cells in the presence of bFGF and cyclopamine (Chandran et al., 2003; Kessaris et al., 2004) have suggested a *Shh*-independent pathway for oligodendrogenesis. However, the efficiency and specificity of the antibody and cyclopamine inhibition could potentially lead to alternative explanations. Our observation that GalC+ oligodendrocytes can develop from *Smo*$^{-/-}$ mutant ES cells provides unambiguous genetic evidence that oligodendrogenesis can occur in the absence of hedgehog signaling (Figure 7), at least in vitro.

Despite the in vitro data for *Shh*-independent oligodendrogenesis, there has been no evidence that this phenomenon can be applied to in vivo development. Our findings that OPCs are generated from *Shh*$^{-/-}$ spinal cord provide the missing link that *Shh*-independent oligodendrogenesis also occurs during spinal cord development as well. In the absence of *Shh* signaling, *Olig1/2*+ OPCs were still generated on schedule (at E14.5) as in the wild-type dorsal spinal cord. Although we can not formally exclude the possibility that the hedgehog signaling in *Shh*$^{-/-}$ mutants could be compensated by the upregulation of expression of other hedgehog members such as *Indian hedgehog* (*Ihh*) or *Desert hedgehog* (*Dhh*), we do not favor this possibility, as we failed to detect by in situ hybridization (ISH) the expression of *Ihh* or *Dhh* in either wild-type or *Shh* mutant spinal cords around the onset of dorsal oligodendrogenesis (data not shown).

The signaling mechanism underlying the *Shh*-independent late phase of dorsal oligodendrogenesis in the spinal cord is uncertain at this stage. Since bFGF can induce oligodendrocytes in dissociated dorsal neural progenitor cells (Gabay et al., 2003) independent of *Shh* signaling (Chandran et al., 2003; Kessaris et al., 2004) and in dorsal explants (our unpublished data), it is conceivable that *FGF* signaling may be partially responsible for the late production of OPCs in the dorsal spinal cord (Figure 9C). In addition, the progressive reduction of *BMP* signaling over time may also contribute to dorsal oligodendrogenesis. It is known that BMP can antagonize *Shh*-induced oligodendrocyte specification, and experimental inhibition of *BMP* signaling is sufficient to induce oligodendrocyte production both in vivo and in vitro (Mekki-Dauriac et al., 2002; Miller et al., 2004; Vallstedt et al., 2005 [this issue of *Neuron*)]. Future studies on the expression and function of various FGF and BMP molecules and their receptors will be needed to determine their possible in vivo roles in the late phase of oligodendrogenesis in the dorsal neural progenitor cells.

Multiple Origins and Phases of Oligodendrogenesis in the Developing Spinal Cord

It is generally accepted that early OPCs are induced from the pMN domain of ventral spinal cord by the *Shh* signal (Poncet et al., 1996; Pringle et al., 1996; Orentas et al., 1999) and that oligodendrocyte development is coupled to motor neuron development (Richardson et al., 2000; Lu et al., 2002; Zhou and Anderson, 2002). However, our data in *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$ mutants and in wild-type embryos as well have provided strong evidence that a subset of OPCs originate from the dorsal spinal cord independent of motor neuron development at later stages of oligodendrogenesis. Therefore, there are multiple origins of, and distinct inductive mechanisms for, OPC production in the developing mammalian spinal cord. Based on these observations, we propose that there are two phases of *Olig* gene expression during normal spinal cord development, the *Shh/Nkx6*-dependent early phase of *Olig* expression and oligodendrogenesis in the pMN domain and the *Shh/Nkx6*-independent late phase of *Olig* expression and oligodendrogenesis in the dorsal spinal cord (Figures 9A and 9B).

The dorsal oligodendrogenesis in mouse spinal cord has long been unnoticed, because the production of OPC cells from the dorsal spinal cord is both late and transient (at around E14.5) as compared to the early OPC production (E12.5) from the ventral spinal cord. By the time OPCs are being generated from the dorsal spinal cord, a large number of ventral-derived OPCs have already invaded into the dorsal spinal cord and thus mask the existence of the late-born dorsal OPCs. Only in mutants (e.g., *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$) in which the ventral oligodendrogenesis from the pMN domain is inhibited or greatly compromised can the dorsal generation of OPCs be uncovered.

Experimental Procedures

Genotyping of *Nkx6* Mutant Mice

The *Nkx6.1* and *Nkx6.2* homozygous null embryos were obtained by the interbreeding of double heterozygous animals. Genomic DNA extracted from embryonic tissues or mouse tails was used for genotyping by Southern analysis or by PCR. Genotyping of *Nkx6.1* and *Nkx6.2* loci was described in Sander et al. (2000) and Cai et al. (2001), respectively. Genotyping of *Shh* mutant mice was carried out according to Chiang et al. (1996).

In Situ RNA Hybridization and Immunohistochemical Staining

Spinal cord tissues at the thoracic level were isolated from E10.5 to E18.5 mouse embryos and then fixed in 4% paraformaldehyde at 4°C overnight. Following fixation, tissues were transferred to 20% sucrose in PBS overnight, embedded in OCT media, and then sectioned (20 μm thickness) on a cryostat. Adjacent sections from the wild-type and mutant embryos were subsequently subjected to ISH or immunofluorescent staining. ISH was performed as described in Schaeren-Wiemers and Gerfin-Moser (1993) with minor modifications, and the detailed protocol is available upon request. Double immunofluorescent procedures were previously described in Xu et al. (2000). For the combination of ISH and immunohistochemistry, sections were first subject to ISH with *Dbx2* riboprobe, rinsed several times with PBS followed by immunohistochemical staining with anti-*Olig2*. Anti-*Pax7* (1:50 DSHB), anti-*Mash1* (1:200), anti-NG2 (1:1500), anti-*PDGFRα* (1:300), anti-*MBP* (1:5000), and anti-GalC (1:50) were obtained from commercial sources. Anti-*Olig2* (1:3000) and anti-*Sox10* (1:3000) polyclonal antibodies were generously provided by Drs. Chuck Stiles and Michael Wegner.

Spinal Cord Explant Culture

Segments of spinal cord tissues were isolated from E11.5, E13.5, or E18.5 embryos at the thoracic region and grown either in collagen gel or on 8.0 μm nucleopore polycarbonate membranes (Costar) floating on culture medium (DMEM + N2 supplement + 30 ng/ml T3 + 40 ng/ml T4 + 1 mg/ml BSA + 0.5% FBS + Pen-Strep). In our experience, the inclusion of a small amount of FBS in culture medium made cells healthier and did not appear to significantly affect oligodendrocyte development in explant culture as compared to no serum (data not shown), although serum was shown to inhibit OPC differentiation (Raff et al., 1983). For anti-*Shh* antibody treatment, 5E1 supernatant (1:3 DSHB) was added to culture medium. Following various days of culture in vitro, explants were then fixed in 4% PFA and processed for immunofluorescent staining (Xu et al., 2000) or whole-mount in situ RNA hybridization with *MBP* riboprobe, as described in Cai et al. (1999).

Culture and Differentiation of Embryonic Stem Cells

Normal and *Smo*$^{-/-}$ ES cells were maintained on MEF feeder cells in ES medium with LIF. During differentiation, ES cells were dissociated and grown on nonadherent petri dishes for 2 days in the absence of LIF and 4 additional days in 5 μM retinoic acid to generate embryoid bodies (EB). Following 10 days of suspension culture, EBs were trypsinized with trypsin/EDTA, and cells were plated on laminin-coated cover slips and cultured for 15 days prior to immunofluorescent staining with anti-GalC antibody.

Acknowledgments

We are grateful to Dr. Andy McMahon for *Smo* mutant ES cells; Dr. Philip Beachy for *Shh* mutant mice; Drs. Chuck Stiles and John Alberta for anti-*Olig2* antibody; and Dr. Michael Wegner for anti-*Sox10* antibody. We also thank Drs. Bill Richardson, Chuck Stiles, and David Anderson for providing cDNA probes; and Dr. Johan Ericson for communicating data prior to publication. This work is supported by the National Multiple Sclerosis Society (RG 3275 for M.Q. and FA1400-A-1 for J.C.) and by NIH (NS37717).

Received: July 16, 2004
Revised: October 7, 2004
Accepted: November 16, 2004
Published: January 5, 2005

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Development 130, 93-102

doi:10.1242/dev.00184

Fgfr3 expression by astrocytes and their precursors: evidence that astrocytes and oligodendrocytes originate in distinct neuroepithelial domains

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Accepted 3 October 2002

SUMMARY

The postnatal central nervous system (CNS) contains many scattered cells that express fibroblast growth factor receptor 3 transcripts (*Fgfr3*). They first appear in the ventricular zone (VZ) of the embryonic spinal cord in mid-gestation and then distribute into both grey and white matter – suggesting that they are glial cells, not neurones. The *Fgfr3*$^+$ cells are interspersed with but distinct from platelet-derived growth factor receptor α (*Pdgfra*)-positive oligodendrocyte progenitors. This fits with the observation that *Fgfr3* expression is preferentially excluded from the pMN domain of the ventral VZ where *Pdgfra*$^+$ oligodendrocyte progenitors – and motoneurones – originate. Many glial fibrillary acidic protein (Gfap)-positive astrocytes co-express *Fgfr3* in vitro and in vivo. *Fgfr3*$^+$ cells within and outside the VZ also express the astroglial marker glutamine synthetase (*Glns*). We conclude that (1) *Fgfr3* marks astrocytes and their neuroepithelial precursors in the developing CNS and (2) astrocytes and oligodendrocytes originate in complementary domains of the VZ. Production of astrocytes from cultured neuroepithelial cells is hedgehog independent, whereas oligodendrocyte development requires hedgehog signalling, adding further support to the idea that astrocytes and oligodendrocytes can develop independently. In addition, we found that mice with a targeted deletion in the *Fgfr3* locus strongly upregulate Gfap in grey matter (protoplasmic) astrocytes, implying that signalling through Fgfr3 normally represses Gfap expression in vivo.

Key words: Fgfr3, Targeted deletion, Astrocyte, Reactive gliosis, CNS, Neuroepithelium

INTRODUCTION

In the embryonic CNS, neurones and glia develop from the neuroepithelial cells of the ventricular zone (VZ) that surrounds the ventricles of the brain and the lumen of the spinal cord. Different domains of the VZ express different gene products and generate different subsets of neurones and/or glia. For example, the ventral half of the spinal cord VZ is subdivided into five regions labelled (from ventral to dorsal) p3, pMN, p2, p1 and p0. These five domains express different combinations of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors and generate distinct classes of spinal neurones; pMN gives rise to somatic motoneurones, whereas p0-p3 give rise to four classes of ventral interneurones (V0-V3 respectively) (reviewed by Briscoe and Ericson, 1999; Jessell, 2001). In the brainstem, p3 also gives rise to visceral motoneurones (Ericson et al., 1997).

After neurones, the VZ switches to producing glial cells. Oligodendrocytes, the myelinating glial cells of the CNS, develop from the ventral VZ. Small numbers of oligodendrocyte progenitors (OLPs), which express the platelet-derived growth factor receptor-α (Pdgfra), first appear at the ventricular surface on embryonic day 12.5 (E12.5) in the mouse, then proliferate and migrate away into the grey and white matter before starting to differentiate into myelin-forming oligodendrocytes (Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). In rodents, OLPs are generated from the same part of the neuroepithelium as somatic motoneurones (MNs) but not until after MN production has ceased (Sun et al., 1998; Lu et al., 2000) (for a review, see Rowitch et al., 2002). This prompted us to suggest that there is a pool of shared neuroglial precursors that first generates MNs, then switches to OLPs (Richardson et al., 1997; Richardson et al., 2000). This idea has been supported recently by the finding that the bHLH proteins Olig1 and Olig2 are expressed and required in pMN for production of both motoneurones and OLPs (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002) (reviewed by Rowitch et al., 2002).

Where do astrocytes, the other major class of CNS glia, originate in the neuroepithelium? It is believed that at least some astrocytes are generated by transdifferentiation of radial

glia (Bignami and Dahl, 1974; Choi et al., 1983; Benjelloun-Touimi et al., 1985; Voigt, 1989; Culican et al., 1990). Others are formed from multipotent precursors in the subventricular zones (SVZ) of the postnatal brain. However, the origins of astrocytes in the developing spinal cord are unclear, so we looked for an astrocyte lineage marker that might be helpful in following the development of astrocytes from their earliest precursors in the VZ. Previous expression studies of the fibroblast growth factor receptor 3 (*Fgfr3*) suggested that this receptor might be expressed in glial cells, possibly astrocytes (Peters et al., 1993; Miyake et al., 1996). Our own studies, reported here, support this conclusion and suggest that *Fgfr3*-positive astrocytes develop from *Fgfr3*-positive precursor cells in the neuroepithelium. *Fgfr3* is not expressed equally in all parts of the neuroepithelium but is reduced or absent from pMN, suggesting that astrocytes and OLPs have separate neuroepithelial origins. We also found that astrocytes are formed in vitro in the absence of hedgehog signalling – unlike oligodendrocytes, which require sonic hedgehog from the ventral midline. This reinforces the notion that at least some astrocytes develop independently of OLPs.

To investigate the function of *Fgfr3* in astrocytes, we examined mice with a targeted deletion in the *Fgfr3* locus (Colvin et al., 1996). The number of *Fgfr3*-expressing cells was normal in the knockout, suggesting that *Fgfr3* does not mediate a mitogenic or survival-promoting effect for these cells. However, Gfap was markedly upregulated in grey matter astrocytes, which normally have little or no Gfap – unlike their counterparts in white matter. Our results imply that signalling through Fgfr3 normally represses Gfap expression in grey matter astrocytes and suggest that white matter astrocytes might preferentially express Gfap because ligands for Fgfr3 are not normally available in axon tracts.

MATERIALS AND METHODS

Tissue and cell cultures

Spinal cords from stage 12-13 (48 hour) chick embryos were dissected into dorsal, middle and ventral thirds using a flame-sharpened tungsten needle. Tissue fragments were cultured as explants in collagen gels (Guthrie and Lumsden, 1994) in defined BS medium (Bottenstein and Sato, 1979) containing 0.25% (v/v) foetal bovine serum (FBS) and conalbumin in place of transferrin (Pringle et al., 1996).

For dissociated cell cultures, E17 rat cervical spinal cords were digested in 0.25% (w/v) trypsin in Earle's buffered saline (Ca^{2+} and Mg^{2+} free; Gibco) for 15 minutes at 37°C, then FBS was added to a final concentration of 10% (v/v) and the tissue physically dissociated by trituration. Cells were washed by centrifugation and resuspended in BS medium before plating in a 50 µl droplet on poly-D-lysine-coated glass coverslips (5×10^4 cells/coverslip). Both explants and dissociated cell cultures were cultured at 37°C in 5% CO_2 in a humidified atmosphere.

Neutralising Shh activity in vitro

Monoclonal Shh neutralising antibody 5E1 (Ericson et al., 1996) was concentrated by ammonium sulphate precipitation from hybridoma supernatants (Harlow and Lane, 1988). Monoclonal anti-NG2 proteoglycan #4.11 (Stallcup and Beasley, 1987) was used as a negative control. Precipitated antibodies were dissolved in a small volume of PBS and dialysed first against PBS and then Dulbecco's modified Eagle's medium (DMEM, Gibco). The final volumes were approximately tenfold less than the starting volumes and were assumed to be ten time as concentrated. Explants were incubated in the presence of either anti-Shh or control antibodies at twice the final concentration. Antibodies were added at the start of the experiment and fresh medium and antibody were added each day thereafter. In some experiments cyclopamine (1 µM; from William Gaffield) instead of anti-Shh was added to cultures daily.

BrdU labelling in vivo

E18 pregnant mice were injected intraperitoneally with BrdU at 50 µg BrdU per gram bodyweight. Two injections were given, 6 hours apart. Mice were sacrificed 3 hours after the second injection and the embryos were processed for *Fgfr3* in situ hybridisation and BrdU immunolabelling.

Preparation of tissue sections

C57Bl/6 mice were obtained from Olac and bred in-house. Noon on the day of discovery of the vaginal plug was designated embryonic day 0.5 (E0.5). We also used *Fgfr3*-null mice (Colvin et al., 1996) bred at UCL. Mid-gestation embryos were staged according to the morphological criteria of Theiler (Theiler, 1972). Rats (Sprague-Dawley) were obtained from the UCL breeding colony and staged according to Long and Burlingame (Long and Burlingame, 1938). Fertilised White Leghorn chicken eggs were obtained from Needle Farm (Cambridge, UK). They were incubated at 38°C and the chicken embryos staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Embryos were decapitated and immersion-fixed in cold 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours before cryoprotecting in cold 20% (w/v) sucrose in PBS for at least 24 hours. In sections processed for immunohistochemistry after in situ hybridisation, the fixation time was reduced to 1 hour to preserve epitope integrity. Tissues were immersed in OCT embedding compound (BDH), frozen on solid CO_2 and stored at –70°C before sectioning. Frozen sections (15 µm) were cut on a cryostat and collected on 3-aminopropyl-triethoxysilane (APES)-coated glass microscope slides. Sections were air-dried for 2 hours before storing dry at –70°C.

Immunohistochemistry

Anti-Gfap monoclonal ascites, clone G-A-5 (Sigma), was used at a dilution of 1:400. Anti-BrdU (monoclonal BU209) (Magaud et al., 1989) was used at 1:5 dilution. Monoclonal O4 (Sommer and Schachner, 1981) was used as cell culture supernatant diluted 1:5. Secondary antibodies were rhodamine- or fluorescein-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin (all from Pierce) diluted 1:200. All antibodies were diluted in PBS containing 0.1% (v/v) Triton X-100 and 10% (v/v) normal goat serum, except O4, which was diluted in PBS alone. Sometimes diaminobenzidine (DAB) labelling (ABC kit, Vector Laboratories) was used instead of fluorescence detection.

In situ hybridisation

Our in situ hybridisation procedures have been described (Pringle et al., 1996; Fruttiger et al., 1999); detailed protocols are available at http://www.ucl.ac.uk/~ucbzwdr/richardson.htm. Digoxigenin (DIG)- or fluorescein (FITC)-labelled RNA probes were transcribed in vitro from cloned cDNAs. The rat *Fgfr3* probe was transcribed from a ~900 bp partial cDNA encoding most of the tyrosine kinase (TK) domain (W.-P. Yu, PhD thesis, University of London, 1995) and the chicken *Fgfr3* probe from a ~440 bp partial cDNA encoding part of the TK domain (from Ivor Mason, King's College London). The mouse *Pdgfra* probe was made from a ~1600 bp cDNA encoding most of the extracellular domain (from Chiayeng Wang, University of Chicago). The chicken *Pdgfra* probe was made from a ~3200 bp cDNA covering most of the 3′ untranslated region of the mRNA (from Marc Mercola, Harvard Medical School, Boston).

Fig. 1. *Fgfr3* expression in transverse sections of embryonic chick and mouse cervical spinal cords. (A) Chick stage 22-24 (E3.5-4); (B) chick stage 34 (E8); (C) chick stage 35 (E9); (D) chick stage 37 (E11); (E) chick stage 35 (E9); (F) mouse E13.5; and (G) mouse E14.5. Initially, *Fgfr3* is expressed in the floor plate and the ventral two-thirds of the VZ (A) and is later downregulated in part of the ventral VZ (B). Starting around stage 34 (E8) *Fgfr3*$^+$ cells are visible in the parenchyma of the cord. By stage 37 (E11) the floor plate and VZ no longer express *Fgfr3* but scattered *Fgfr3*$^+$ cells are present throughout most of the cross-section of the cord, including both grey and white matter (D). (E) A magnified image of the ventral VZ from a stage 35 (E9) cord, showing the two spatially separated domains of *Fgfr3* expression. A similar progression occurs in mouse (F,G). However, the ventral 'gap' is not so pronounced in mouse (arrow in G). Scale bars: 200 μm (A-D), 100 μm (F,G), 50 μm (E).

For double in situ hybridisation, two probes – one FITC labelled and the other DIG labelled – were applied to sections simultaneously. The FITC signal was visualised with alkaline phosphatase (AP)-conjugated anti-FITC Fab_2 fragments before developing in p-iodonitrotetrazolium violet (INT) and 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) (BCIP), which produces a magenta/brown reaction product. The sections were photographed, then the AP was inactivated by heating at 65°C for 30 minutes followed by incubating in 0.2 M glycine (pH 2) for 30 minutes at room temperature. The INT-BCIP reaction product was removed by dehydration through graded alcohols, concluding with 100% ethanol for 10 minutes at room temperature. The DIG signal was then visualised with AP-conjugated anti-DIG Fab_2 fragments and a mixture of nitroblue tetrazolium (NBT) and BCIP (all reagents from Roche Molecular Biochemicals) and the sections re-photographed. No labelling with NBT/BCIP was observed when we omitted either the DIG labelled probe or the anti-DIG antibody (data not shown).

For the *Fgfr3-Pdgfra* double in situ hybridisation of Fig. 4 we visualised the FITC (*Pdgfra*) signal with horseradish peroxidase (HRP)-conjugated anti-FITC Fab_2 fragments (Roche) before developing in fluorescein-tyramide reagent (NEN™ Life Science Products, Boston) according to the manufacturer's instructions. The HRP-conjugate was inactivated by incubating in 2% (v/v) hydrogen peroxide for 30 minutes at room temperature. The DIG (*Fgfr3*) signal was then visualised with HRP-conjugated anti-DIG Fab_2 fragments followed by rhodamine-tyramide, and the sections photographed under fluorescence optics. As specificity controls we omitted either the FITC-labelled *Pdgfra* probe or the HRP-conjugated anti-FITC antibody, which gave no staining other than for *Fgfr3* (not shown).

Combined immunolabelling and in situ hybridisation

For the experiment of Fig. 7, cultured cells were first subjected to in situ hybridisation with a [^{35}S]-labelled RNA probe against *Fgfr3* then immunolabeled with anti-Gfap and biotinylated goat-anti-mouse Ig. The Gfap signal was developed with DAB and the slides dehydrated through ascending alcohols, dipped in nuclear emulsion (Ilford K5), exposed in the dark for several days and developed in Kodak D19.

RESULTS

Fgfr3 expression in the embryonic spinal cord

We examined *Fgfr3* expression in the embryonic chick spinal cord by in situ hybridisation. At stage 22-24 (corresponding to ~E4), *Fgfr3* expression was confined to the floor plate and the ventral two-thirds of the VZ (Fig. 1A). By stage 34 (E8) *Fgfr3* expression had been extinguished in part of the ventral VZ so that a gap developed in the expression pattern (e.g. Fig. 1B).

Individual *Fgfr3*$^+$ cells were also present outside the VZ

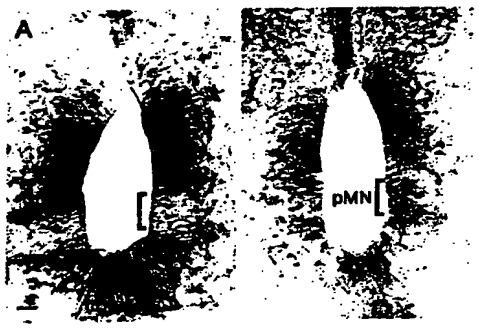

Fig. 2. Expression of *Fgfr3* and *Olig2*. Transverse sections through stage 35 (E9) chicken spinal cords were subjected to in situ hybridisation for *Fgfr3* (A) or double in situ for *Fgfr3* and *Olig2* (B). At this age, *Fgfr3* expression is confined to the VZ and a few scattered cells outside the VZ. The two spatially separated domains of *Fgfr3* expression are clearly visible (A). *Olig2* is expressed predominantly within the ventral 'gap' of *Fgfr3* expression (B). This suggests that pMN (brackets), which generates *Pdgfra*$^+$ oligodendrocyte progenitors (OLPs), does not also generate *Fgfr3*$^+$ putative astrocyte progenitors. Scale bar: 50 μm.

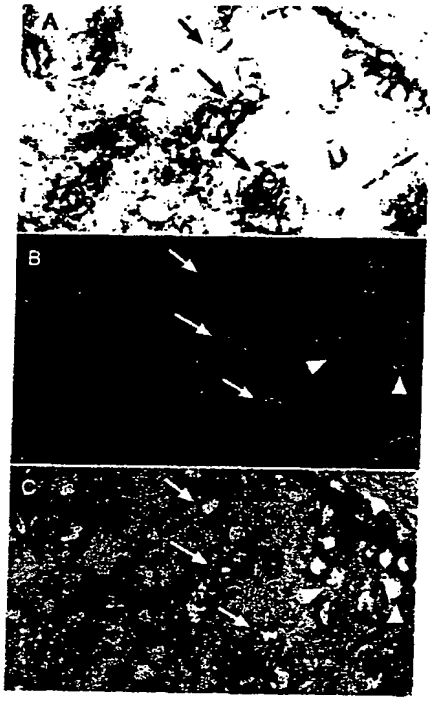

Fig. 3. Incorporation of BrdU by *Fgfr3*-expressing cells. We labelled E18 embryos by two intra-peritoneal injections of BrdU, 6 hours apart, into the mother. We harvested the embryos 3 hours later and performed in situ hybridisation for *Fgfr3* followed by immunohistochemistry for BrdU. The *Fgfr3* (A) and BrdU (B) images were superimposed using Adobe Photoshop (C). Many *Fgfr3*-expressing cells incorporated BrdU (C, arrows), confirming that they can divide after exiting the VZ and are therefore unlikely to be neurones. Arrowheads in B,C indicate *Fgfr3*-negative cells that have incorporated BrdU.

after stage 34 (E8), both lateral and dorsal to the *Fgfr3*$^+$ neuroepithelial domains. Often the individual cells appeared to be streaming away from the VZ into the parenchyma. This is evident in Fig. 1C, for example. By stage 37 (E11) *Fgfr3* expression was no longer detectable in the VZ but scattered *Fgfr3*$^+$ cells were present throughout the grey and white matter of the cord (Fig. 1D). *Fgfr3* expression followed a similar progression in mouse and rat (Fig. 1F,G and not shown). In rodents, however, the ventral gap in *Fgfr3* expression was not as pronounced as in chicks (Fig. 1G, arrow).

A scattered population of *Fgfr3*-expressing cells is found throughout most regions of the late embryonic and postnatal mouse brain, both in white and in grey matter. As in the embryonic spinal cord, there appear to be specific regions of the embryonic brain VZ that give rise to *Fgfr3*$^+$ cells that stream away from the VZ into the parenchyma (not shown).

Fgfr3-expressing cells originate mainly outside the pMN domain of the neuroepithelium

In the developing spinal cord, neuroepithelial precursors at different positions along the dorsoventral axis generate distinct neuronal subtypes. The ventral half of the spinal cord VZ is

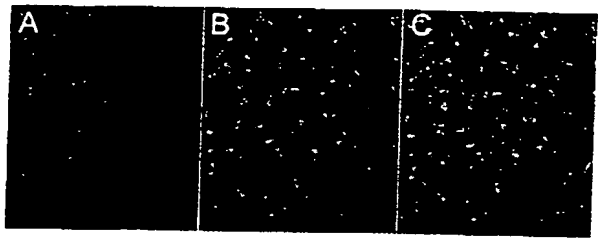

Fig. 4. Different populations of *Fgfr3*$^+$ and *Pdgfra*$^+$ cells in the newborn spinal cord. We hybridised sections of P2 mouse cervical spinal cord simultaneously with a DIG-labelled *Fgfr3* probe together with an FITC-labelled *Pdgfra* probe to visualise OLPs. The *Fgfr3* signal (red) was visualised with rhodamine-tyramide reagent and the *Pdgfra* signal (green) with fluorescein-tyramide. Scattered individual *Fgfr3*$^+$ and *Pdgfra*$^+$ cells can be seen throughout both white and grey matter of the cord, but these are separate and discrete cell populations. We conclude that the great majority of *Fgfr3*$^+$ cells in the cord are not OLPs.

divided into five neuroepithelial domains known as (from ventral to dorsal) p3, pMN, p2, p1 and p0 (Briscoe et al., 2000). Of these, pMN is known to generate motoneurones followed by oligodendrocyte progenitors (OLPs). It seemed to us that the ventral gap in *Fgfr3* expression (Fig. 2A) might correspond to pMN. To test this, we performed double in situ hybridisation for *Fgfr3* and *Olig2* (which defines pMN) (Lu et al., 2000; Zhou et al., 2000). At stage 35, the *Olig2* in situ hybridisation signal was within the gap in the *Fgfr3* signal (Fig. 2B, arrow). Therefore, *Fgfr3* is preferentially downregulated in pMN where oligodendrocyte lineage cells originate, but is expressed both ventral and dorsal to pMN.

Fgfr3- expressing cells are glia

The fact that most of the scattered *Fgfr3*$^+$ cells are generated after stage 34 (E8) in the chick, E13.5 in mouse, is itself a strong argument that they are glial cells, not neurones, because most spinal neurones are born before this (Altman and Bayer, 1984). That some of the *Fgfr3*$^+$ cells are found in axon tracts also suggests that they are glia, for there are very few neuronal cell bodies in fibre tracts.

Another indication that they are glial cells is that they continue to divide after they leave the VZ. We showed this by injecting BrdU into a pregnant mouse at18 days gestation. The embryos were removed 3 hours later and processed by in situ hybridisation for *Fgfr3* followed by immunolabelling for BrdU. We found many (*Fgfr3*$^+$, BrdU$^+$) cells scattered throughout the white and grey matter of the cord (Fig. 3, arrows). This confirms that *Fgfr3*-expressing cells divide in vivo and are therefore unlikely to be neurones or neuronal progenitors, which leave the VZ as postmitotic cells. This strengthens the idea that the *Fgfr3*-expressing cells are glia. There was also a population of (BrdU$^+$, *Fgfr3*$^-$) cells in both grey and white matter (Fig. 3C, arrowheads), so there is a distinct population(s) of dividing cells that do not express *Fgfr3*.

Fgfr3-expressing cells are distinct from Pdgfra$^+$ oligodendrocyte progenitors

To determine whether the *Fgfr3*$^+$ cells that we detect are oligodendrocyte progenitors (OLPs), we double labelled

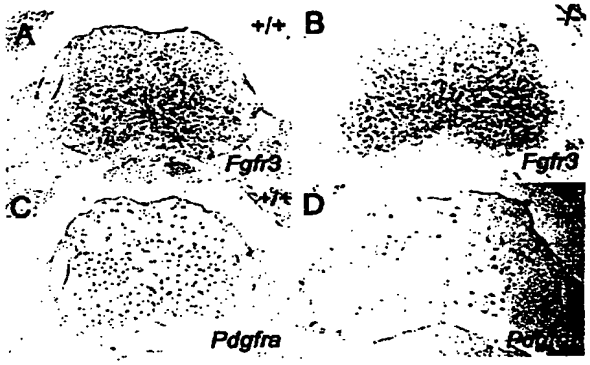

Fig. 5. *Fgfr3*-positive cells are unaffected in *Pdgfa* null spinal cords. Consecutive sections of newborn wild-type or *Pdgfa* knockout mouse cervical spinal cords were hybridised in situ with probes to *Fgfr3* (A,B) or *Pdgfra* (C,D). The number of *Pdgfra*$^+$ OLPs is strongly reduced in the *Pdgf-A* knockout (compare C with D) but neither the number nor the distribution of *Fgfr3*$^+$ cells is changed noticeably (A,B). Again, we conclude that the *Fgfr3*$^+$ cells and *Pdgfra*$^+$ OLPs are different cells.

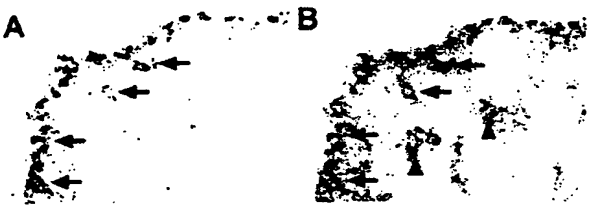

Fig. 6. Newly differentiating white matter astrocytes express *Fgfr3*. We simultaneously hybridised sections of E18 mouse cervical spinal cord with an FITC-labelled *Gfap* mRNA probe (A) and a DIG-labelled *Fgfr3* probe (B). The *Gfap* and *Fgfr3* hybridisation signals were visualised and photographed sequentially (see Materials and Methods). All the *Gfap*-expressing astrocytes also expressed *Fgfr3* (e.g. arrows). In general, *Fgfr3*$^+$ cells in the grey matter (arrowheads) did not co-express *Gfap*.

mouse E18 and P2 spinal cord sections for *Fgfr3* and *Pdgfra*, an established marker of early OLPs. Both in situ hybridisation probes labelled similar numbers of cells that were scattered throughout the spinal cord grey and white matter, but the two cell populations were completely non-overlapping (Fig. 4). This also held true throughout the postnatal brain (N. P. P., unpublished). We also looked in newborn *Pdgfa* knockout mice which contain far fewer *Pdgfra*$^+$ OLPs than normal (Fruttiger et al., 1999). Despite the lack of OLPs, there were normal numbers of *Fgfr3*$^+$ cells at this age (Fig. 5). Clearly, the *Fgfr3*$^+$ cells detected by our in situ hybridisation procedures are not early OLPs but a different cell population. This is consistent with the fact that in mice lacking Fgfr3, early events of oligodendrocyte lineage progression occur normally and the numbers of *Pdgfra*$^+$ cells remains unchanged (R. Bansal, personal communication) (N. P. P., unpublished).

Fgfr3-expressing cells are astrocytes and astrocyte precursors

To test whether the *Fgfr3*$^+$ cells might be astrocytes, we double labelled E18 mouse spinal cord sections for *Fgfr3* and *Gfap* mRNAs. At E18, white matter astrocytes begin to express *Gfap* mRNA, which initially remains in the astrocyte cell bodies and allows identification of individual astrocytes. (As astrocytes mature further, both *Gfap* mRNA and protein are relocated to the extending cell processes, making individual cells difficult to distinguish.)

All the *Gfap*$^+$ astrocytes in developing white matter at E18 also expressed *Fgfr3* (Fig. 6, arrows). This result clearly identifies many of the *Fgfr3*-expressing cells as astrocytes. Nevertheless, the majority of *Fgfr3*-expressing cells in the grey matter (Fig. 6B, arrowheads) are *Gfap*-negative. We presume that these represent *Gfap*-negative, possibly immature, astrocytes.

In an attempt to label all astrocytes, including *Gfap*-negative astrocytes, we used an in situ hybridisation probe against glutamine synthetase mRNA (*Glns*) (EC 6.3.1.2). Glns is widely regarded as an astrocyte marker, although there have been reports that it is also present in mature oligodendrocytes and even OLPs. We found that *Glns* transcripts were present in the VZ of the E15 mouse spinal cord and in cells outside the VZ in a pattern that was very similar that of *Fgfr3* (Fig. 7). This is consistent with the view that *Fgfr3* and *Glns* mark astrocytes and their precursors. This conclusion was further strengthened by studies of cultured astrocytes (see below).

Cultured astrocytes co-express Gfap and Fgfr3

When CNS cells are dissociated and placed in culture, astrocytes in the culture upregulate Gfap and are easily recognisable. We dissociated and cultured cells from E17 rat cervical spinal cord and labelled them by in situ hybridisation for *Fgfr3* and by immunocytochemistry for Gfap. Almost all of the Gfap$^+$ astrocytes also expressed *Fgfr3* (Table 1; Fig. 8, arrows). There was also a small population of flat, fibroblast-like *Fgfr3*$^+$ cells that did not express Gfap (Fig. 8, arrowheads). The number of these cells decreased with time in culture; at 3 days they were 6% of all cells, by 9 days less than 1% (Table 1). These (*Fgfr3*$^+$, Gfap$^-$) cells might be astrocyte precursors or immature astrocytes that have not yet upregulated Gfap. In any

Table 1. E17 rat spinal cord cell cultures double labelled for *Fgfr3* and Gfap

Days in vitro	*Fgfr3*$^+$ Gfap$^-$ (astrocyte precursors?)	*Fgfr3*$^+$ Gfap$^+$ (astrocytes)	*Fgfr3*$^-$ Gfap$^+$ (astrocytes)	*Fgfr3*$^-$ Gfap$^-$ (other cells)
3	17/275 (6%)	69/275 (25%)	None	189/275 (68%)
6	23/596 (4%)	82/596 (14%)	2/596 (<1%)	489/596 (82%)
9	4/645 (<1%)	197/645 (31%)	1/645 (<1%)	443/645 (69%)

Dissociated cells from E17 rat spinal cord were cultured for 3, 6 or 9 days and then subjected to in situ hybridization for *Fgfr3* mRNA followed by immunohistochemistry for Gfap protein. We counted astrocytes (*Fgfr3*$^+$, Gfap$^+$ and *Fgfr3*$^-$, Gfap$^+$), putative astrocyte precursors (*Fgfr3*$^+$, Gfap$^-$) and other unidentified cells (*Fgfr3*$^-$, GFAP$^-$). The great majority of GFAP-expressing astrocytes also expressed *Fgfr3*. These data are from a single representative experiment (duplicate coverslips); comparable results were obtained in two additional independent experiments.

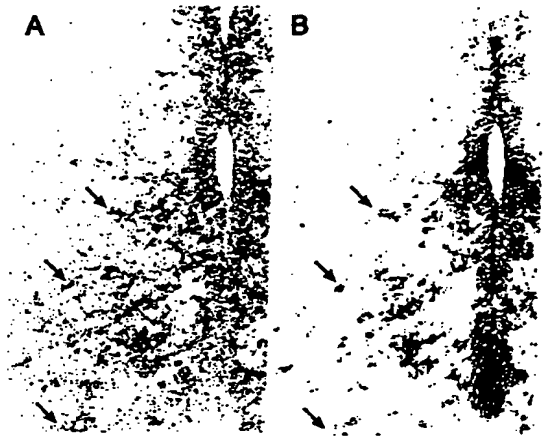

Fig. 7. Co-expression of *Fgfr3* and glutamine synthetase (*Glns*) in the VZ and parenchyma of the embryonic mouse spinal cord. There was considerable overlap between the in situ hybridisation signals for *Fgfr3* and *Glns* in the E15 mouse spinal cord, strongly suggesting that *Fgfr3*$^+$ cells correspond to glial (presumably astrocyte) precursors. Arrows indicate cells that express both *Fgfr3* and *Glns*.

case, this experiment provides clear evidence that most or all Gfap$^+$ astrocytes in culture co-express *Fgfr3*.

Gfap is upregulated in grey matter astrocytes in Fgfr3 null mice

If *Fgfr3* is expressed by astrocytes, we might expect to see specific effects on astrocytes in transgenic mice homozygous for a targeted disruption of *Fgfr3*. These mice have previously been shown to have skeletal and inner ear defects but no CNS defects have yet been reported (Colvin et al., 1996).

We visualised astrocytes in spinal cord sections of 3-month-old *Fgfr3* null mice, together with their heterozygous *Fgfr3*$^{+/-}$ and wild-type littermates, by immunolabelling with anti-Gfap. Heterozygous and null mutant mice all displayed the normal pattern of Gfap expression up to 6 weeks of age. Gfap expression was observed in the white matter around the circumference of the spinal cord, many Gfap-labelled processes being oriented in a radial direction (Fig. 9A). By comparison, there was little or no Gfap expression in the grey matter, except in astrocytes associated with blood vessels. Between 6 weeks and 2 months of age, a striking up-regulation of Gfap expression occurred in the grey matter of *Fgfr3* null mice, though not in their heterozygous or wild-type littermates (Fig. 9B). Astrocytes lining blood vessels also had increased Gfap immunoreactivity.

The number of cells that contain *Fgfr3* mRNA was not noticeably different in *Fgfr3*-null spinal cords compared with wild type (data not shown). This suggests that Fgfr3 does not normally mediate a signal for proliferation or survival of astrocytes, although further experiments (e.g. BrdU labelling in vivo) would be required to substantiate this.

Astrocyte development in vitro does not depend on Hedgehog signalling

In the spinal cord, production of ventral cell types – motoneurones, ventral interneurones and OLPs – is dependent

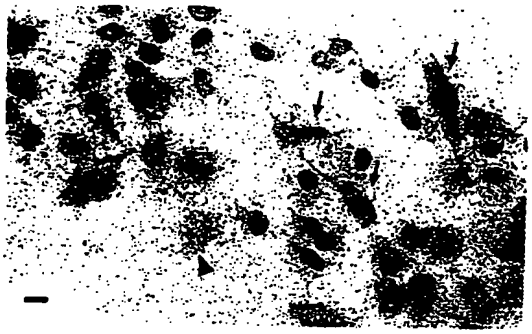

Fig. 8. Cultured cells from E17 rat spinal cord double-labelled for *Fgfr3* and Gfap. Cells were hybridised in situ with a ^{35}S-labelled RNA probe for *Fgfr3*, then immunolabelled for Gfap followed by autoradiography (see Materials and Methods). The *Fgfr3* signal (black silver grains) is present over most Gfap-positive cells (brown DAB reaction product; arrows) (also see Table 1). Scale bar: 10 μm. Arrowhead indicates an *Fgfr3*-positive, *Gfap*-negative cell.

on Shh signalling (Ericson et al., 1996; Orentas et al., 1999) (for a review, see Jessell, 2001). We wanted to know whether production of astrocytes from the ventral neural tube is also dependent on Shh. We microdissected stage 12/13 (E2) chick spinal cord into thirds along the dorsoventral axis and cultured the ventral-most fragments in collagen gels with either a control antibody or an anti-Shh neutralising antibody (see Materials and Methods). After 48 hours in culture we labelled explants with monoclonal antibody 4D5, which recognises homeodomain proteins Isl1 and Isl2 in motoneurones. Control explants contained numerous Isl$^+$ cells, whereas none were observed in explants incubated with anti-Shh (data not shown). After a further 10 days in culture (12 days total) we visualised OLPs with monoclonal antibody O4 (Sommer and Schachner, 1981) (Fig. 10C,D) and astrocytes with anti-Gfap (Fig. 10A,B). All of the explants incubated with control antibody (19/19) contained large numbers (>300) of O4$^+$ late-stage OLPs (Fig. 10C). As expected, OLP production was markedly decreased by anti-Shh (Fig. 10D); 14/22 explants contained no O4$^+$ cells and, of the remaining eight explants, seven contained fewer than ten positive cells and the other one contained 38 positive cells. By contrast, all explants contained numerous (>300) Gfap$^+$ astrocytes whether they had been incubated with control antibody (19/19) or anti-Shh (22/22) (Fig. 10A,B).

Similar results were obtained with explants from stage 25 (E5) embryos from which we were able to dissect the ventral one-quarter of the neural tube and discard the floor plate. Once again, large numbers of Gfap$^+$ astrocytes developed in explants cultured with control antibody (22/22) and with anti-Shh (25/25), even though OLP production in these explants was inhibited by anti-Shh (not shown).

To test the possibility that other hedgehog (Hh) proteins (Desert Hh, Indian Hh) control astrocyte production in ventral explants, we inhibited the activity of all isoforms with the alkaloid cyclopamine (Cooper et al., 1998; Incardona et al., 1998). This gave similar results as Shh neutralising antibodies (data not shown). Thus, we conclude that astrocyte induction in ventral spinal cord does not require Hedgehog signalling.

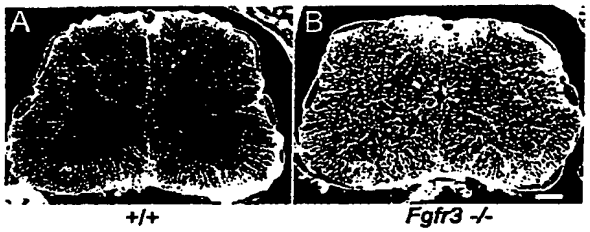

Fig. 9. Gfap upregulation in grey matter astrocytes in *Fgfr3*-null mice. Transverse sections through the cervical spinal cords of 2-month-old wild-type (A) and *Fgfr3*-null mice (B) were immunolabeled with anti-Gfap. In the wild-type cord, white matter (fibrous) astrocytes express Gfap but there is little or no Gfap immunoreactivity in the grey matter. By contrast, the *Fgfr3*-null mouse (B) shows extensive Gfap labelling of grey matter (protoplasmic) astrocytes. Scale bar: 100 μm.

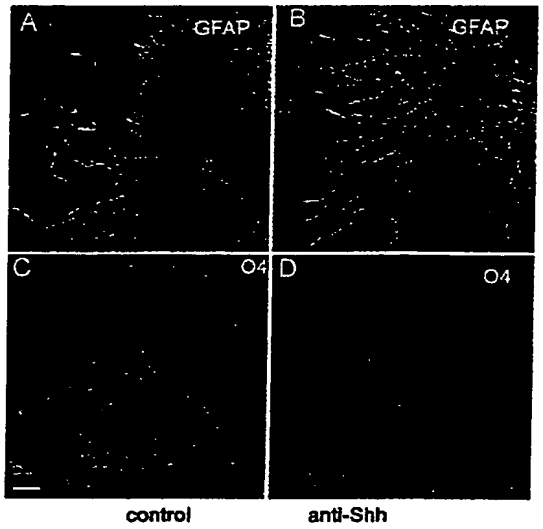

Fig. 10. Neutralising *Shh* activity in explant cultures of ventral spinal cord. Stage 12/13 (E2) chick neural tube was dissected into dorsal, intermediate and ventral thirds. The ventral thirds were cultured in collagen gels in the presence of either control antibody (A,C) or with neutralising anti-Shh antibody (B,D). Explants were double-labelled with O4 monoclonal antibody (C,D) and anti-Gfap (A,B). Anti-Shh blocks the formation of O4-positive OLPs but not Gfap-positive astrocytes. Scale bar: 10 μm.

DISCUSSION

On the basis of their spatial distribution and time of appearance, Peters et al. (Peters et al., 1993) suggested that *Fgfr3*$^+$ cells in the mouse CNS are glial cells, possibly astrocytes. By double labelling experiments with *Fgfr3* and Gfap, Miyake et al. (Miyake et al., 1996) concluded that *Fgfr3* was expressed in astrocytes in the adult rat brain. Our data support and extend these conclusions. We present evidence that scattered *Fgfr3*$^+$cells in the embryonic and postnatal CNS are astrocytes and/or astrocyte progenitors, and that these astrocytes are derived from *Fgfr3*$^+$ neuroepithelial precursors in the VZ.

Fgfr3 is also expressed transiently by a subpopulation of motoneurones (Philippe et al., 1998) and by late oligodendrocyte progenitors (late OLPs) just prior to terminal differentiation in vitro (Bansal et al., 1996). We are convinced that the *Fgfr3*$^+$ cells that we detect are not OLPs, however. First and foremost, double labelling for *Fgfr3* and *Pdgfra* (a marker of early OLPs) demonstrates that these mark separate populations of cells. The *Fgfr3*$^+$ and *Pdgfra*$^+$ cell populations appear at different times and initially their distributions are different. Moreover, the number and distribution of *Fgfr3*$^+$ cells was unaltered in neonatal *Pdgfa*-null spinal cords, which have very few *Pdgfra*$^+$ OLPs and oligodendrocytes (Fruttiger et al., 1999). This argues strongly that the large majority of *Fgfr3*$^+$cells revealed by our in situ hybridisation protocol are not OLPs. Bansal et al. (Bansal et al., 1996) have shown that OLPs do express *Fgfr3* mRNA in culture but only at a low level during the earlier stages of the lineage. Presumably this is below our limit of detection in situ. OLPs upregulate *Fgfr3* strongly just prior to oligodendrocyte differentiation (Bansal et al., 1996) but these presumably represent a small subset of OLPs in the embryonic spinal cord and do not feature in our analysis.

Fgfr3-positive cells co-expressed mRNA encoding glutamine synthetase (Glns; EC 6.3.1.2). In the CNS, Glns is an accepted marker of mature astrocytes (Norenberg and Martinez-Hernandez, 1979; Stanimirovic et al., 1999) but it is also expressed in oligodendrocytes (Domercq et al., 1999) and OLPS (Baas et al., 1998). Glns has not previously been ascribed to neuroepithelial precursors or immature astrocytes in the embryo, although *Glns* transcripts have been detected in the rat brain by northern blot as early as E14. To our knowledge, Glns has not been described in neurones except in pathological situations such as Alzheimer's disease (Robinson, 2000). Therefore, we are confident that the (*Fgfr3*$^+$, *Glns*$^+$) double-positive cells described here are glial cells. Taken together with the evidence against them being OLPs (see above), it seems likely that they correspond to immature and mature astrocytes. This is strongly supported by the observations that *Fgfr3*$^+$ cells co-express Gfap protein and/or mRNA in (1) the formative white matter of the normal developing spinal cord and (2) cultures of dissociated spinal cord cells.

Neuroepithelial origins of astrocytes

Fgfr3 was expressed in two domains of the spinal cord neuroepithelium separated by an *Fgfr3*-negative region. This was true of both rodent and avian embryos though it was more obvious in the latter. The *Fgfr3*-negative region corresponds roughly to the pMN domain of the VZ that generates somatic motoneurones followed by *Pdgfra*$^+$ OLPs (Sun et al., 1998; Rowitch et al., 2002). Therefore, our data indicate that OLPs and astrocytes originate from separate precursors that reside in different parts of the VZ. How does this fit with other ideas about the origin of astrocytes? One hypothesis is that astrocytes arise by transdifferentiation of radial glia, after the latter have fulfilled their role as cellular substrates for radial migration of neuronal progenitors (Bignami and Dahl, 1974; Choi et al., 1983; Benjelloun-Touimi et al., 1985; Voigt, 1989; Culican et al., 1990). This could be compatible with our *Fgfr3* expression data, as radial glia have their cell bodies close to the ventricular

surface. However, radial glia are distributed all around the spinal cord lumen, unlike *Fgfr3*, so one would have to postulate that only a subset of radial glia express *Fgfr3*.

In double-knockout mice that lack the two basic helix-loop-helix (bHLH) transcriptions factors Olig1 and Olig2, the pMN domain of the VZ undergoes a homeotic transformation into p2, its immediate dorsal neighbour (Rowitch et al., 2002). As a result, pMN no longer generates motoneurones followed by OLPs, but instead produces V2 interneurones followed by astrocytes (Zhou and Anderson, 2002; Takebayashi et al., 2002). By implication, this is the usual fate of p2 precursors in wild-type mice. This is consistent with our observation that *Fgfr3*$^+$ astrocytes apparently originate within an extended part of the ventral VZ, including p2 but excluding pMN. Our *Fgfr3* expression data are also consistent with previous fate mapping experiments in chick-quail chimeras, which indicated that astrocytes are generated from dorsal as well as ventral parts of the VZ, whereas OLPs are generated only from ventral territory (Pringle et al., 1998). It remains to be seen whether astrocytes that are generated from distinct neuroepithelial domains (p3 or p2, say) have identical properties or whether they are functionally specialised – for modulating synaptic activity or interacting with blood vessels, for example.

Production of ventral cell types such as motoneurones, interneurones and OLPs is dependent on Shh signalling. As many *Fgfr3*-expressing astrocyte precursors appear to originate in p3, p2 and other ventral domains, we might expect that production of astrocytes might also depend on Shh signalling. However, we found that astrocytes developed in explant cultures of ventral neural tube either in the presence or absence of Shh activity. Our data imply that astrocytes are specified by different mechanisms than OLPs – at least, they demonstrate that astrocyte and OLP production are not obligatorily linked. In fact, there is evidence that more than one signalling pathway can lead to astrocyte development in vitro (Rajan and McKay, 1998). Because astrocytes can be formed from dorsal as well as ventral neuroepithelium, it remains possible that 'ventral' astrocytes might normally be under Shh control, but that by blocking Shh signalling we uncover an alternative 'dorsal' pathway for astrocyte development.

It has been reported that there are glial-restricted precursor cells (GRPs) in the embryonic rat spinal cord that are dedicated to the production of astrocytes and oligodendrocytes (Rao and Mayer-Proschel, 1997; Herrera et al., 2001). This seems to conflict with current evidence that oligodendrocytes and astrocytes are generated from different precursors in the embryonic spinal cord (Lu et al., 2002; Rowitch et al., 2002; Zhou and Anderson, 2002) (this paper). A possible reconciliation might be that GRPs with the potential to generate both astrocytes and oligodendrocytes are formed in all parts of the spinal cord VZ but are constrained in vivo to generate only astrocytes or only oligodendrocytes, depending on the signals in their local environment (i.e. where they are located) (for a review, see Rowitch et al., 2002).

Fgfr3 regulates Gfap expression in grey matter astrocytes

Astrocytes with distinct, heritable morphologies have been described in cultures of rat spinal cord cells (Fok-Seang and Miller, 1992). Astrocytes in different parts of the CNS differ in morphology or function in vivo too, suggesting that they might fulfil different, region-specific functions. In addition, astrocytes in white matter tracts generally have smaller cell bodies with more and longer processes compared to their counterparts in grey matter (Connor and Berkowitz, 1985). For this reason, white matter astrocytes are sometimes referred to as 'fibrous' and those in grey matter as 'protoplasmic' or 'velous'. White matter astrocytes also express high levels of Gfap, whereas grey matter astrocytes contain little or no immunoreactive Gfap.

Fibrous and protoplasmic astrocytes might develop from separate lineages (Connor and Berkowitz, 1985). However, our observation that Gfap is upregulated in grey matter astrocytes of *Fgfr3*-null mice provides strong in vivo evidence that extracellular signals might be required to maintain their normal Gfap-negative phenotype. This is consistent with a report that adding Fgf2 to cultured astrocytes downregulates Gfap mRNA and protein and causes their morphology to change (Reilly et al., 1998). Fgf2 and other known Fgfr3 ligands such as Fgf9 are made by, and presumably released from, many CNS neurones (Eckenstein et al., 1991; Cotman and Gomez-Pinilla, 1991; Woodward et al., 1992; Gomez-Pinilla et al., 1994; Kuzis et al., 1995). One possible reason that white matter astrocytes express high levels of Gfap in wild-type mice might be that they are denied exposure to *Fgfr3* ligands in axon tracts – perhaps because Fgf, like Pdgf, is secreted from neuronal cell bodies but not from axons (Fruttiger et al., 2000).

Upregulation of Gfap in the *Fgfr3*-null mouse is mindful of the astrocyte response to CNS injury or disease – so-called reactive gliosis or astrocytosis (for reviews, see Ridet et al., 1997; Norton, 1999). It would be interesting to know whether interruption of signalling through Fgfr3 is somehow involved in the astrocyte reaction to injury. However, it is unlikely to be straightforward, because Gfap upregulation in the *Fgfr3*-null animals does not occur until around 2 months of age, suggesting that it is an indirect effect. In addition, the data from the *Fgfr3*-null mouse are difficult to square with the observation that intra-ventricular injection of Fgf2 has been reported to increase the number of Gfap$^+$ reactive astrocytes (Unsicker, 1993).

Most grey matter (protoplasmic) astrocytes possess many short sheet-like processes containing little, if any, Gfap (Connor and Berkowitz, 1985). It has been suggested that this morphology might help them to infiltrate the neuropil and surround axonal terminals, synapses and neuronal cell bodies, consistent with one of their proposed roles in neurotransmitter metabolism (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979). It will be interesting to see if the reactive astrocytes in *Fgfr3*-null mice are defective in neurotransmitter metabolism and whether this contributes to the premature death of the animals.

We thank our colleagues for advice and discussions, Marc Mercola, Ivor Mason, Martin Raff, Chiayeng Wang and William Gaffield for reagents, and Damith Jayatilake, Xiang Hua and Ed Spinaio for technical help. Work in W. D. R.'s laboratory was supported by the UK Medical Research Council and the European Union (QLRT-1999-31556 and QLRT-1999-31224). Work in D. M. O.'s laboratory was supported by NIH grant CA60673. We also thank Rashmi Bansal for sharing unpublished data.

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The Journal of Neuroscience, January 1, 2002, 22(1):248–256

The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal–Ventral Differences in GRP Cell Function

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We have found that the tripotential glial-restricted precursor (GRP) cell of the embryonic rat spinal cord can give rise *in vitro* to bipotential cells that express defining characteristics of oligodendrocyte-type-2 astrocyte progenitor cells (O2A/OPCs). Generation of O2A/OPCs is regulated by environmental signals and is promoted by platelet-derived growth factor (PDGF), thyroid hormone (TH) and astrocyte-conditioned medium. In contrast to multiple observations indicating that oligodendrocyte precursor cells in the embryonic day 14 (E14) spinal cord are ventrally restricted, GRP cells are already present in both the dorsal and ventral spinal cord at E13.5. Ventral-derived GRP cells, however, were more likely to generate O2A/OPCs and/or oligodendrocytes than were their dorsal counterparts when exposed to TH, PDGF, or even bone morphogenetic protein-4. The simplest explanation of our results is that oligodendrocyte generation occurs as a result of generation of GRP cells from totipotent neuroepithelial stem cells, of O2A/OPCs from GRP cells and, finally, of oligodendrocytes from O2A/OPCs. In this respect, the responsiveness of GRP cells to modulators of this process may represent a central control point in the initiation of this critical developmental sequence. Our findings provide an integration between the earliest known glial precursors and the well-studied O2A/OPCs while opening up new questions concerning the intricate spatial and temporal regulation of precursor cell differentiation in the CNS.

Key words: glial-restricted precursor cell; GRP cell; oligodendrocyte; O2A progenitor cell; OPCs; spinal cord development; ventral origin; neuroepithelial stem cells

Understanding how the differentiated cell types of the body are generated is a central challenge in developmental biology. Multiple components contribute to this process, including signaling molecules and transcription factors that cause precursor cells to progress along different developmental pathways. Central to understanding cell generation, however, is identification of the precursor cell from which a given cell type arises, for it is the specific precursor cell that represents the actual target for exogenous influences.

The creation of specific precursor cells and differentiated cell types proceeds through a sequence of lineage restrictions but also may involve a phenomenon of lineage convergence. Through lineage restriction, the totipotent stem cells of the earliest embryo generate progeny that are more restricted in the range of cell types they generate. For example, totipotent embryonic stem cells give rise to tissue-specific stem cells. Tissue-specific stem cells proceed to produce differentiated cell types via intermediate lineage-restricted precursor cells. These lineage-restricted precursor cells ultimately generate a subset of the differentiated cell types in a particular tissue. Lineage restriction is complemented in development by the process of lineage convergence, by which different lineages give rise to the same cell type. One example of such convergence is seen in the formation of cartilage from both mesenchymal and cranial neural crest lineage (Baroffio et al., 1991).

Studies on CNS development are revealing a rich diversity of precursor cells that can give rise to the same cell type, particularly with respect to glial development. For example, it is well established that oligodendrocytes can be generated from oligodendrocyte-type-2 astrocyte progenitor cells (Raff et al., 1983; Skoff and Knapp, 1991), which also are referred to as oligodendrocyte precursor cells (Raff et al., 1983; Skoff and Knapp, 1991) and abbreviated here as O2A/OPCs. More recent studies on embryonic rat spinal cord have led to the isolation of a new and distinct population, called tripotential glial-restricted precursor (GRP) cells, that also can generate oligodendrocytes *in vitro* and *in vivo* (Rao et al., 1998; Herrera et al., 2001). GRP cells and O2A/OPCs differ in several characteristics. For example, GRP cells and O2A/OPCs differ in their responses to mitogens, survival factors, and inducers of differentiation (Rao et al., 1998). GRP cells and O2A/OPCs also express distinct differentiation potentials *in vitro*: GRP cells are able to generate oligodendrocytes and two distinct astrocyte populations, whereas O2A/OPCs can generate oligodendrocytes and only one kind of astrocyte. Moreover, GRP cells readily generate astrocytes when transplanted into the neonatal or adult brain (Herrera et al., 2001), a cell type not generated from primary O2A/OPCs, after transplantation into the normal CNS (Espinosa de los Monteros et al., 1993).

Several critical questions arise from the fact that it now is possible to isolate two distinct precursor cell populations (i.e., GRP cells and O2A/OPCs) from the developing animal, each of

Received June 28, 2001; revised Oct. 10, 2001; accepted Oct. 19, 2001.

This work was supported by a Howard Hughes fellowship award (N.G.), by the Multiple Sclerosis Society (M.M.-P.), and by National Institutes of Health Grant NS37166 (M.N.). We thank all members of our laboratories for enthusiastic and helpful discussions and also thank Samuel Bernard and David Gass for excellent technical assistance.

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Table 1. Differentiation potential of GRP-derived O4$^+$ and O4$^-$ cells

	O4$^+$ cells		O4$^-$ cells	
Culture condition	PDGF/TH	10% FCS	PDGF/TH	10% FCS
	Number of clones that contain specific cell types/number of all scored clones			
Type-2 astrocytes only	0/15	21/22	0/29	1/21
Type-1 astrocytes only	0/15	1/22	0/29	3/21
Type-1 and type-2 astrocytes	0/15	0/22	0/29	17/21
Oligodendrocytes and progenitors	15/15	0/22	29/29	0/21

Individual GRP cell-derived O4$^+$ and O4$^-$ cells were expanded to a clonal size of 5–10 cells before being exposed to 10% FCS to induce astrocytic differentiation or exposed to PDGF and TH to promote oligodendrocyte differentiation. After 5 or 10 d, respectively, clones were stained with anti-GFAP, A2B5, and anti-GalC antibodies. In serum-containing medium, all but one of the clones derived from O4$^+$ cells contained only astrocytes with the antigenic phenotype of type-2 astrocytes (i.e., GFAP$^+$ and A2B5$^+$). In contrast, clones generated from O4$^-$ cells contained a mixture of type-1 (i.e., GFAP$^+$A2B5$^-$) and type-2 astrocytes. Although some clones derived from O4$^+$ or O4$^-$ cells contained one or two progenitor cells, none of the clones contained oligodendrocytes at that time point. All clones exposed to PDGF and TH, regardless of being derived from O4$^+$ or O4$^-$ cells, contained A2B5$^+$ progenitor cells and GalC$^+$ oligodendrocytes. None of these clones contained astrocytes.

which can generate oligodendrocytes. Is the relationship between these two populations one of lineage restriction or lineage convergence? If GRP cells and O2A/OPCs are related, what signals promote the generation of one from the other and how can the existence of both populations be integrated with existing studies on the generation of oligodendrocytes during spinal cord development?

MATERIALS AND METHODS

Cell culture. A2B5$^+$ GRP cells were isolated from embryonic day 13.5 (E13.5) Sprague Dawley rat spinal cords by positive selection on immunopanning dishes coated with A2B5 antibody (Rao et al., 1998). GRP cells were then grown in the presence of 10 ng/ml basic FGF (bFGF) and indicated supplements for various time points on fibronectin/laminin-coated coverslips at 3000 cells/well for mass culture experiments or on coated grid dishes for clonal analysis. Cultures were fed every other day with the factors indicated. At the end of the experiment, cells were stained with O4 (Sommer and Schachner, 1981) or A2B5 antibodies to detect precursor cells, anti-galactocerebroside (GalC) antibody (Gard and Pfeiffer, 1990; Gard et al., 1995) to identify oligodendrocytes, and anti-GFAP antiserum to identify astrocytes (Norton and Farooq, 1993; Morita et al., 1997; Gomes et al., 1999) followed by appropriate fluorochrome-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL). The number of cells of each type relevant to each experiment was calculated, as was the total cell number. As originally defined, GFAP$^+$ cells were scored as type-2 astrocytes if they were stellate and A2B5$^+$ and as type-1 astrocytes if they were fibroblast-like in morphology and were A2B5$^-$.

Rationale for use of the O4 antibody in analyzing generation of O2A/OPCs from GRP cells. To determine whether one cell type gives rise to another, it is useful to identify a marker that is expressed by one cell type but not by the other. This is particularly problematic for analysis of GRP cells and O2A/OPCs. Freshly isolated GRP and O2A/OPCs both label with the A2B5 monoclonal antibody. We have shown previously that GRP cells can express receptors for platelet-derived growth factor (PDGF) without losing their tripotentiality (Rao et al., 1998). Our ongoing studies have revealed that tripotential GRP cells also label with anti-GD3 and anti-NG-2 antibodies (C. Pröschel, D. Gass, and M. Mayer-Pröschel, unpublished observations). Thus, none of these markers, which have been used by many others to study development of O2A/OPCs (Hart et al., 1989; Yim et al., 1995; Nishiyama et al., 1996), allow a distinction to be made between GRP cells and O2A/OPCs.

At this stage, the only remaining candidate marker for investigating whether GRP cells can generate O2A/OPCs is the O4 monoclonal antibody (Sommer and Schachner, 1981). This antibody can be used to define a secondary stage of O2A/OPC development, in which A2B5$^+$O4$^-$ O2A/OPCs give rise to cells that are A2B5$^+$ and also O4$^+$. The great majority of O2A/OPCs isolated from the p7 optic nerve are O4$^+$ (M. Noble, unpublished observations), whereas GRP cells are O4$^-$ (Rao and Mayer-Pröschel, 1997; Rao et al., 1998). In addition, it has been shown that development of GalC$^+$ oligodendrocytes in the O2A/OPC lineage is preceded by the appearance of cells that are O4$^+$ but GalC$^-$ (Schachner et al., 1981; Sommer and Schachner, 1981; Bansal et al., 1989; Gard and Pfeiffer, 1990, 1993). Critically, O4$^+$GalC$^-$ cells isolated from many regions of the postnatal CNS, including spinal cord, are bipotential cells capable of differentiating into both oligodendrocytes and type-2 astrocytes (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999). O4$^+$GalC$^-$ cells also can be induced to proliferate *in vitro* and in this respect are not terminally differentiated (Small et al., 1987; Trotter et al., 1989; Gard and Pfeiffer, 1990; Reynolds and Wilkin, 1991; Warrington and Pfeiffer, 1992; Avossa and Pfeiffer, 1993; Barnett et al., 1993; Gard et al., 1995). Thus, although some authors have preferred to consider O4$^+$GalC$^-$ cells (isolated from postnatal animals or derived from O2A/OPCs) as more committed "oligodendroblasts," the O4$^+$GalC$^-$ cells studied thus far express those characteristics (in particular, bipotentiality *in vitro* and ability to divide) that are most important in defining a cell as being a bipotential O2A/OPC.

Clonal analysis of E13.5 GRP cell-derived O4$^+$ cells. We confirmed the differentiation potential of a cell by clonal differentiation analysis, as used in our previous studies on GRP cells (Rao and Mayer-Pröschel, 1997; Rao et al., 1998) and extensive studies on O2A/OPCs (Ibarrola et al., 1996; Smith et al., 2000); this is the only technique that allows the differentiation characteristics of individual precursor cells to be unambiguously ascertained. The basic strategy used to conduct such analyses in the present studies was as follows: GRP cells were isolated from E13.5 spinal cord as described previously and grown either for 24 hr or for 21 d in the presence of bFGF (10 ng/ml) before being exposed to the condition most effective at generating O4$^+$GalC$^-$ cells (i.e., chemically defined medium supplemented with 10 ng/ml PDGF-A chain homodimer; Peprotech, Rocky Hill, NJ) and thyroid hormone (TH; Sigma, St. Louis, MO). It is critical to note that GRP cells grown for 24 hr in FGF do not express PDGF receptor-α (PDGFR-α), whereas long-term cultured GRP cells express this receptor. We have determined that when grown in the presence of FGF, GRP cells remain tripotential regardless of their PDGF receptor status (Rao et al., 1998). After periods of additional *in vitro* growth indicated in Results, cultures were labeled with both O4 and anti-GalC antibodies, followed by appropriate fluorescein- and rhodamine-conjugated secondary antibodies. Fluorescence-activated cell sorting was then used to obtain populations of O4$^+$GalC$^-$ cells. O4$^+$GalC$^-$ cells were plated at clonal density and single O4$^+$GalC$^-$ cells were identified and circled. Cells were induced to divide for 5 d (in PDGF/bFGF at 10 ng/ml), and clones were switched to PDGF plus TH or 10% FCS when they reached a density of 5–10 cells. After 10 or 3 d, respectively, clones were stained with the A2B5, anti-GFAP, and anti-GalC antibodies. Control cells were switched to PDGF plus TH or 10% FCS without previous proliferation and stained after an additional 10 and 3 d, respectively. The results of our clonal analyses are shown in Tables 1 and 2.

Immunostaining of clones. Staining procedures were as described previously (Rao and Mayer-Pröschel, 1997). Briefly, the A2B5 and anti-GalC antibodies were grown as hybridoma supernatants (American Type Culture Collection, Manassas, VA) and used at a dilution of 1:2. The O4 hybridoma cell line was a generous gift from Ilse Sommer (University of Glasgow, Glasgow, UK), and its supernatant was also used at a 1:2 dilution. Anti-GFAP (polyclonal, rabbit anti-cow; purchased from Dako, Glostrup, Denmark) was used at a 1:100 dilution and applied overnight. All secondary antibodies [i.e., goat anti-mouse IgM-biotin, IgG3-tetramethylrhodamine B isothiocyanate, goat-anti-rabbit Ig (heavy and light chain)-FITC (Southern Biotechnology), and streptavidin (Molecular Probes, Eugene, OR)] were used at a 1:100 dilution. Anti-NG2

Table 2. NSC-derived A2B5$^+$ cells represent GRP cells

Antigenic phenotypes of cells within clones	Ventral E10.5 spinal cord-derived					Dorsal E10.5 spinal cord-derived				
	GFAP$^+$ A2B5$^+$ and GFAP$^+$ A2B5$^-$	GFAP$^+$ A2B5$^-$ only	GFAP$^+$ A2B5$^-$ only	A2B5$^+$ only	GalC$^+$	GFAP$^+$ A2B5$^+$ and GFAP$^+$ A2B5$^-$	GFAP$^+$ A2B5$^-$ only	GFAP$^+$ A2B5$^+$ only	A2B5$^+$ only	GalC$^+$
Percentage of clones (total number) after 7 d in 10% FCS/bFGF	70 (59)	0	0	30 (26)	0	83 (68)	0	0	17 (14)	0
Percentage of clones (total number) after 10 d in TH/bFGF	29 (33)	0	0	36 (40)	35 (39)	34 (23)	0	0	41 (29)	25 (18)

NSCs were isolated from dorsal and ventral regions of the E10.5 spinal cord and expanded in nondifferentiation conditions. After 3 d, dorsal and ventral cultures were allowed to differentiate, and the appearing A2B5$^+$ cells were harvested and replated at clonal density. Expanded clones were exposed either to 10% FCS or to TH to promote astrocytic and oligodendrocytic differentiation, respectively (both conditions also contained FGF). Both ventral- and dorsal-derived clones generated two types of astrocytes in the presence of 10% FCS and GalC$^+$ oligodendrocytes in the presence of TH. All clones that contained oligodendrocytes also contained A2B5$^+$ progenitor cells. We did not see any clones that contained only one type of astrocytes. The numbers shown refer to the number of clones containing different cell types but not the relative composition of the entire culture. For example, the great majority of astrocyte-containing clones in TH only had 1–5% GFAP$^+$ cells, suggesting that the presence of FGF allows some astrocyte differentiation to occur even when TH is present. Numbers in parentheses refer to the total number of clones, whereas numbers without parentheses indicate the percentage of clones in each category.

antiserum was a generous gift from Dr. W. Stallcup (Burnham Institute, La Jolla Cancer Research Center, CA) and was used at a 1:100 dilution.

RESULTS

Tripotential GRP cells, which are O4$^-$ cells, generate bipotential O4$^+$GalC$^-$ cells when grown in the presence of PDGF and thyroid hormone

The first question we addressed was whether tripotential GRP cells can generate cells with the antigenic and differentiation characteristics of bipotential O2A/OPCs. This question was investigated by a combined analysis of antigen expression and of differentiation potential at the clonal level. The requirement to use the O4 antibody (Sommer and Schachner, 1981) as a potential marker of O2A/OPCs is explained in Materials and Methods. Briefly, both GRP cells and O2A/OPCs label with the A2B5 antibody, the NG-2 antibody (Stallcup and Beasley, 1987), and the anti-GD3 antibody (Seyfried and Yu, 1985), and both populations can express PDGF receptors while maintaining their characteristic differentiation potential. Thus, of all of the markers that have been used to study the ancestors of oligodendrocytes, it was only the O4 antibody that remained potentially useful in this context. We designed experiments that would allow us to answer the following questions: (1) are there *in vitro* growth conditions that promote the generation of O4$^+$GalC$^-$ cells from O4$^-$ GRP cells, and (2) do GRP cell-derived O4$^+$GalC$^-$ cells still behave like tripotential GRP cells or do they now behave like bipotential O2A/OPCs?

We first examined the effects on GRP cells of a wide variety of conditions (see Materials and Methods) shown previously to induce generation of oligodendrocytes in cultures of O2A/OPCs. Although astrocyte-conditioned medium in combination with TH was the most effective condition for inducing the appearance of oligodendrocytes over a 3 d time period (data not shown), it was growth in the presence of FGF plus PDGF plus TH that was associated with the generation of the greatest proportion of O4$^+$GalC$^-$ cells.

In cultures of freshly isolated GRP cells that were grown for 24 hr in the presence of FGF and then additionally exposed to PDGF plus TH (with FGF still present), 78 ± 9% of the cells were O4$^+$GalC$^-$ after 3 d in culture. In addition, we noticed that 20 ± 5% of all cells were O4$^+$GalC$^+$ oligodendrocytes and a small percentage (2 ± 0.7%) of cells represented GFAP$^+$ astrocytes. We never observed the appearance of any cells that were GalC$^+$ but O4$^-$, consistent with previous observations that passage through an O4$^+$ stage is required before the expression of GalC immunoreactivity (Schachner et al., 1981; Sommer and Schachner, 1981; Bansal et al., 1989; Gard and Pfeiffer, 1990, 1993). GRP cell cultures that were grown in the presence of FGF alone contained no O4$^+$ cells, and previous studies have demonstrated that GRP cells expanded in this manner retain the ability to generate oligodendrocytes, type-1 astrocytes, and type-2 astrocytes.

Although previous studies have shown that O4$^+$GalC$^-$ cells isolated from postnatal animals or derived from bipotential O2A/OPCs are bipotential *in vitro* (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999), it cannot be assumed that such differentiation characteristics necessarily apply to O4$^+$GalC$^-$ cells derived from tripotential GRP cells. To determine the differentiation potential of GRP cell-derived O4$^+$GalC$^-$ cells, we cultured expanded GRP cells in the presence of FGF for several days, grew them in the additional presence of PDGF plus TH for 3 more days, purified the O4$^+$GalC$^-$ cells, and analyzed their differentiation potential in clonal cultures. Extending the previous expansion period in FGF in this manner resulted in a higher percentage of the cells in the culture remaining O4$^-$, thus allowing the study of this population also.

Cloned O4$^+$GalC$^-$ cells derived from GRP cells expressed the bipotential differentiation characteristics associated with O2A/OPCs. When grown in conditions that induced generation of astrocytes, O4$^+$GalC$^-$ cells derived from GRP cells exhibited the typical differentiation response of O2A/OPCs. In the presence of 10% FCS, the only astrocytes generated in 21 of 22 clones derived from O4$^+$GalC$^-$ cells were type-2 astrocytes (i.e., A2B5$^+$GFAP$^+$ stellate cells; Table 1 and Fig. 1*A*). Only one clone generated type-1-like astrocytes (i.e., A2B5$^-$GFAP$^+$ cells with a fibroblast-like morphology), a frequency low enough to be consistent with the possibility that this one clone had been mislabeled at the beginning of the experiment. This outcome was very different from that obtained with GRP cells themselves, clones of which generate a combination of type-1 and type-2 astrocytes in these conditions (Rao et al., 1998). Moreover, the O4$^-$GalC$^-$ cells that remained after the purification process were still tripotential, emphasizing that the acquisition of bipo-

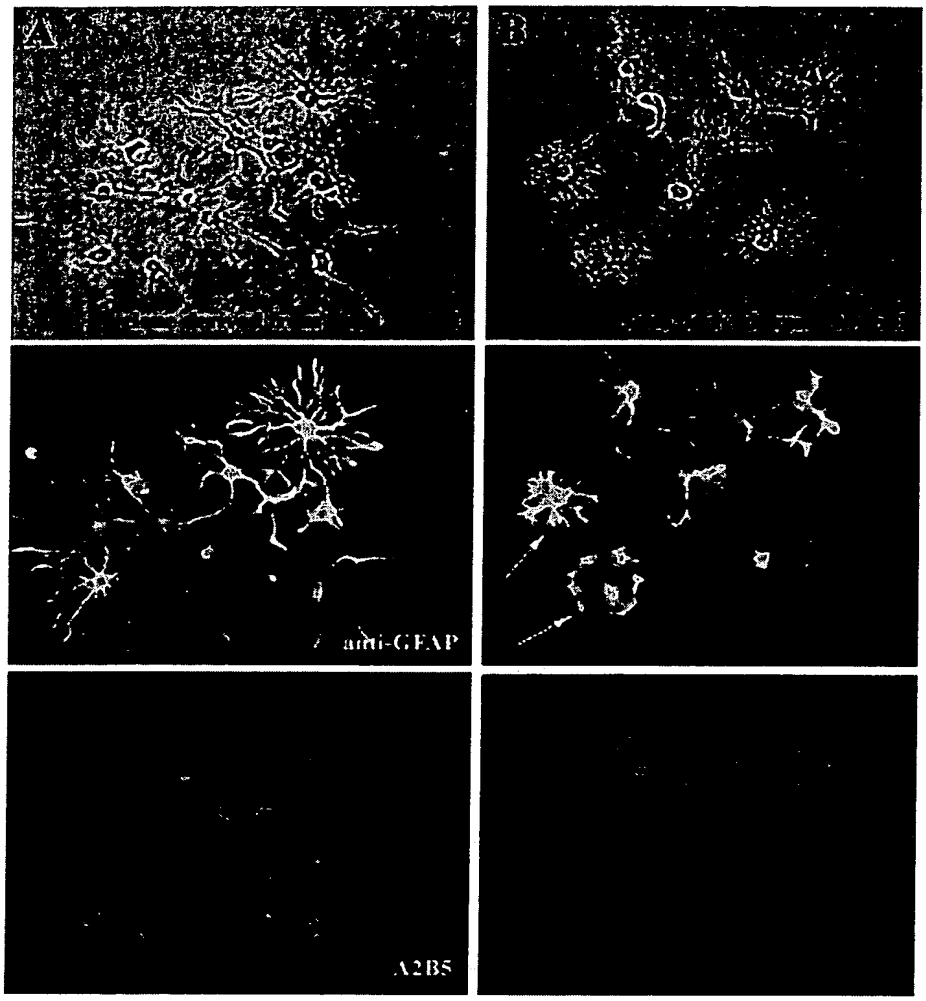

Figure 1. GRP-derived O4$^+$ cells are bipotential and represent O2A/OPC-like cells. Freshly isolated GRP cells were grown for 3 weeks in defined medium in the presence of bFGF and then switched to a medium supplemented with bFGF and TH; after 5 d, cultures were stained with the O4 antibody (see Materials and Methods). Cells were then dislodged from the surface and plated at clonal density in poly-L-lysine-coated dishes. Single O4$^+$ cells were circled. After 3 d in culture, cells were exposed to medium supplemented with bFGF and 10% FCS. (Parallel experiments using BMP4 instead of FCS yielded identical results.) After 5 d, clones were stained with A2B5 (rhodamine), anti-GFAP (fluorescein), and anti-GalC (coumarin) antibodies. The coumarin staining is not shown because none of the clones contained any GalC$^+$ oligodendrocytes in this condition. *A*, Clone derived from a single O4$^+$GalC$^-$ cell. The progeny from O4$^+$ founder cells consists exclusively of A2B5/GFAP double-positive cells, consistent with the antigenic phenotype of type-2 astrocytes. *B*, Clone derived from a single O4$^-$GalC$^-$ cell. The progeny from O4$^-$ founder cells consists of A2B5/GFAP double-positive type-2 astrocytes and A2B5$^-$GFAP$^+$ cells, representing type-1 astrocytes (indicated by *arrows*).

tentiality was a specific event and not merely associated with aging, even in conditions that promote the transition to a bipotential phenotype. When grown in the presence of 10% FCS, O4$^-$GalC$^-$ cells generated clones containing a mixture of type-1 and type-2 astrocytes (Table 1 and Fig. 1*B*), and thus behaved as GRP cells. In contrast to this difference with respect to astrocyte induction, both O4$^+$GalC$^-$ and O4$^-$GalC$^-$ cell-derived clones contained oligodendrocytes when grown in the presence of PDGF plus TH.

The simplest explanation of the data obtained in the above experiments is that GRP cells can generate O4$^+$GalC$^-$ cells that exhibit *in vitro* the defining bipotential differentiation restriction of O2A/OPCs. The results also indicate that generation of such bipotential cells is an environmentally regulated differentiation event, for which PDGF and TH represent potent inducing agents.

GRP cells can be isolated from both ventral and dorsal E13.5 spinal cord

A critical component of the current understanding of oligodendrocyte development *in vivo* is that specific precursor cells for oligodendrocytes first appear in the ventral spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Timsit et al., 1995; Hall et al., 1996; Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). We subsequently determined whether GRP cells are selectively localized in the ventral spinal cord at or before the time when putative oligodendrocyte precursor cells first appear ventrally. Because previous studies have shown that GRP cells are already present at E13.5 (Rao et al., 1998), we microdissected dorsal and ventral portions of the E13.5 cord to determine the regional distribution of GRP cells; this is a half day earlier than the earliest reported appearance of specific oligodendrocyte precursor cells, as defined by expression of the PDGF receptor (Hall et al., 1996). Freshly isolated cells from dorsal and ventral cord were immunolabeled with A2B5 antibody, purified by fluorescence-activated cell sorting, and plated at clonal density on grid dishes in different conditions as described below. In three independent experiments, the dorsal spinal cord consistently contained an average of 19 ± 8% A2B5$^+$ cells, whereas the ventral portion contained an average of 52 ± 7% A2B5$^+$ cells. Thus, although the ventral cord contained a higher proportion of A2B5$^+$ cells than did the dorsal cord, such cells were found in both regions of the cord.

To determine whether dorsal- and ventral-derived A2B5$^+$ cells

were GRP cells, the A2B5$^+$ clones were first grown in the presence of bFGF until they reached a size of 10–20 cells. Astrocytic differentiation was then induced by exposing cultures for 3 d to 10% FCS. All clones contained both A2B5$^-$GFAP$^+$ type-1 astrocytes and A2B5$^+$GFAP$^+$ type-2 astrocytes independent of their site of isolation. Thus, these cells were typical of GRP cells in their ability to generate two distinct astrocyte populations. Generation of oligodendrocytes was also possible with both ventral- and dorsal-derived cells, as discussed in the following section.

GRP cells derived from both the ventral and dorsal E13.5 spinal cord can generate O2A/OPCs, oligodendrocytes, and astrocytes

Because expression of PDGF receptor-α in the E14 spinal cord has been interpreted to be an indication of a preferential ventral origin of oligodendrocytes (Pringle and Richardson, 1993; Hall et al., 1996), we asked whether ventral- and dorsal-derived GRP cells differed in their ability to generate O2A/OPCs and/or oligodendrocytes. GRP cells were isolated from ventral or dorsal E13.5 spinal cord as described in the preceding section. Freshly isolated cells were plated at a low density on coverslips in the presence of FGF and exposed to conditions (PDGF plus TH) that would induce the transition into O2A/OPCs (as determined previously) or to conditions that would potentially inhibit a transition into O2A/OPCs. As a potential inhibitor molecule, we used bone morphogenetic protein-4 (BMP4), which has been shown to inhibit oligodendrocyte generation (Mabie et al., 1997; Grinspan et al., 2000; Mehler et al., 2000; Zhu et al., 2000) and is present in the embryonic neural tube (D'Alessandro and Wang, 1994; Barth et al., 1999; Grinspan et al., 2000; Liem et al., 2000). After 3 d of *in vitro* growth in the condition discussed, cells were stained with the O4 monoclonal antibody and with anti-GalC and anti-GFAP antibodies.

In the presence of PDGF and TH, cells from both the dorsal and ventral spinal cord were able to generate O4$^+$GalC$^-$ cells with equal frequencies but differed with respect to oligodendrocyte generation (Fig. 2). Specifically, 88 ± 6% of dorsal-derived cells were O4$^+$GalC$^-$, 3 ± 2% were GalC$^+$ oligodendrocytes, and 3 ± 2% were GFAP$^+$ astrocytes. In contrast, 74 ± 9% of ventral-derived cells were O4$^+$GalC$^-$, 28 ± 5% were GalC$^+$ oligodendrocytes, and 2 ± 1% were GFAP$^+$ astrocytes. Thus, although both dorsal and ventral cells were able to generate O2A/OPCs, only ventral-derived cells generated a significant number of GalC$^+$ oligodendrocytes over a 5 d time period. The lack of oligodendrocytes in dorsal cultures is not likely to be attributable to preferential cell death. because the total number of cells was not different in dorsal and ventral cultures (327 ± 8 and 326 ± 38. respectively). In addition, dorsal-derived cells demonstrated an equal ability to eventually generate oligodendrocytes. If cultures were examined after 10 d in the presence of TH, instead of after 5 d, then 69 ± 15% of the ventral cells and 73 ± 3% of dorsal cells were oligodendrocytes (data not shown).

Differences between dorsal- and ventral-derived GRP cells were also observed in response to BMP4. When dorsal- or ventral-derived GRP cells were grown in the presence of BMP4 in concentrations ranging from 1 to 100 ng/ml over 3 d, we observed that BMP4 promoted the generation of astrocytes in both dorsal and ventral cells (Fig. 3). At a low BMP concentration (1 ng/ml), ventral cells were more likely to differentiate into astrocytes than were dorsal cells (45 ± 4% vs 14 ± 4%, respectively). The preferential generation of GFAP$^+$ cells in ventral

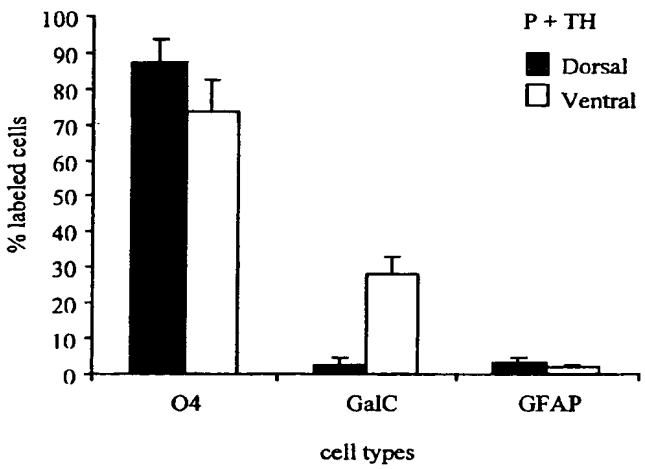

Figure 2. Both dorsal- and ventral-derived GRP cells generate O4$^+$GalC$^-$ cells. A2B5$^+$ cells were isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos and plated in the presence of bFGF supplemented with PDGF plus TH (*P + TH*) for 7 d. Dorsal and ventral cultures were then stained with O4, anti-GalC, and anti-GFAP antibodies. Both dorsal- and ventral-derived cultures generated comparable numbers of O4$^+$ precursor cells. However, GalC$^+$ oligodendrocytes were found predominantly in ventral-derived GRP cell cultures. Only a small fraction of both dorsal and ventral cultures gave rise to GFAP$^+$ astrocytes. Two independent experiments examining six data points for each condition revealed comparable results.

cultures was a transient phenomenon, in that only in dorsal-derived cultures did these numbers increase over the next several days (as discussed in the following paragraph). The addition of 10 ng/ml BMP had an identical effect on ventral and dorsal cells (48 ± 3% and 54 ± 7% astrocytes, respectively). The most dramatic difference between ventral and dorsal cells was observed at high BMP doses (100 ng/ml). In this condition, ventral cells responded with cell death rather than cell differentiation. In contrast, dorsal-derived GRP cells differentiated almost completely into astrocytes when exposed to 100 ng/ml BMP4.

Because BMP4 at 1 ng/ml revealed differences between dorsal- and ventral-derived GRP cells in the absence of toxicity, we subsequently examined the generation of O4$^+$GalC$^-$ cells in this culture condition (Fig. 4*A*). Cells were plated at a low density on coverslips in the presence of FGF and BMP4 (1 ng/ml) and examined after 7 d to allow for the generation of O4$^+$GalC$^-$ cells and/or GalC$^+$ oligodendrocytes. In cultures of GRP cells derived from dorsal spinal cord, the majority of cells (87 ± 8%) differentiated into GFAP$^+$ astrocytes, and only 12 ± 7% of the cells were O4$^+$GalC$^-$. We did not observe any GalC$^+$ oligodendrocytes in these cultures. In contrast, when ventral-derived cells were exposed to 1 ng/ml BMP for 7 d, 47 ± 8% differentiated into astrocytes (as observed for 3 d time point discussed previously) and 52 ± 7% of the cultures consisted of O4$^+$GalC$^-$ cells. Again, we did not observe any GalC$^+$ oligodendrocytes. Thus, BMP4 exposure was associated with a strikingly more significant decrease in the number of O4$^+$GalC$^-$ cells in dorsal- than in ventral-derived GRP cells.

We subsequently determined whether the addition of TH, a potent inducer of the generation of O4$^+$GalC$^-$ cells and/or oligodendrocytes, could counteract the effects of BMP4 (Fig. 4*B*). Dorsal and ventral cells were exposed to BMP at 1 ng/ml in the presence of TH at 50 nM for 7 d before the cultures were labeled

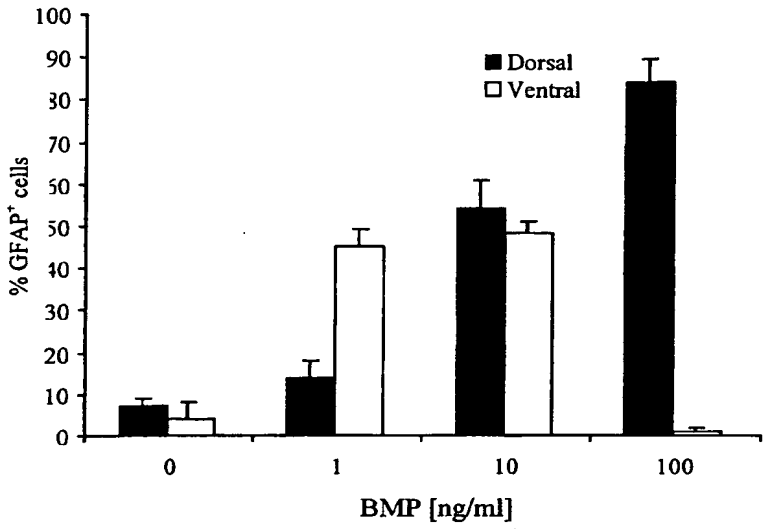

Figure 3. BMP4 induces differentiation of astrocytes from dorsal- and ventral-derived A2B5$^+$ cells in a dose-dependent manner. A2B5$^+$ cells, isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos, were plated at a low density in the presence of bFGF and increasing concentrations of BMP4 (0.1–100 ng/ml). After 3 d, cultures were labeled with anti-GFAP antibodies and the number of astrocytes was determined. Whereas dorsal cultures exhibited a continuous, dose-dependent increase in the number of GFAP$^+$ astrocytes, ventral-derived GRP cells generated significantly more astrocytes at lower doses of BMP (1 ng/ml) at this time point, and higher doses of BMP4 (100 ng/ml) proved to be lethal to ventral-derived GRP cells.

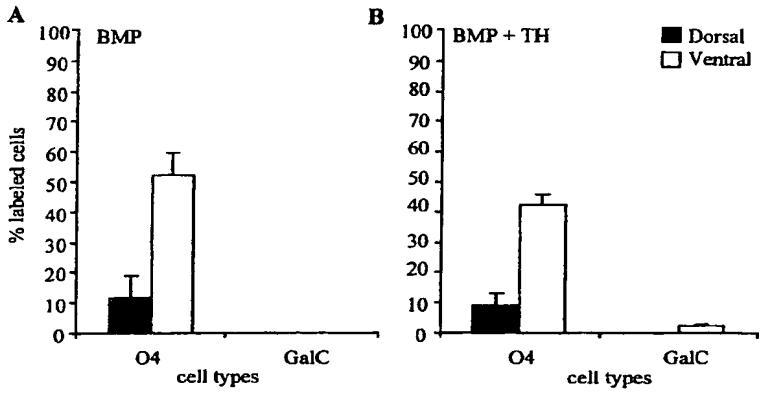

Figure 4. Differential effects of BMP4 on dorsal- and ventral-derived GRP cells. GRP cells were isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos and plated at a low density in the presence of FGF and BMP4 (*A*) (1 ng/ml) or FGF, BMP4 (1 ng/ml), and TH (*B*). To allow for oligodendrocyte generation, cultures were examined after 7 d for the presence of O4$^+$GalC$^-$ precursor cells or GalC$^+$ oligodendrocytes. Although GalC$^+$ oligodendrocytes were only found in ventral GRP cell cultures containing TH, both dorsal- and ventral-derived cultures contained O4$^+$GalC$^-$ precursor cells. In the presence of BMP, the ability of dorsal GRP cells to generate O4$^+$GalC$^-$ precursor cells was lower than that of ventral-derived cultures; this was not changed by the addition of TH.

with O4, anti-GalC, and anti-GFAP antibodies. As shown in Figure 4*B*, the addition of TH had little or no effect on the generation of O4$^+$GalC$^-$ cells in both dorsal and ventral cultures. However, we did detect a small but significant increase ($p < 0.002$) in the number of GalC$^+$ oligodendrocytes specifically in the ventral-derived cells. This effect was not seen in dorsal-derived cultures.

GRP cells can be generated from dorsal and ventral neuroepithelial stem cells of the E10.5 spinal cord

Our results thus far demonstrate that there is a dorsal–ventral gradient in GRP cell distribution in the spinal cord of the E13.5 rat, and that dorsal- and ventral-derived GRP cells are dissimilar in their abilities to generate oligodendrocytes over short time periods *in vitro*. In addition, these two populations differ in their response to BMP. Because GRP cells themselves are derived from neuroepithelial stem cells (NSCs) (Rao and Mayer-Pröschel, 1997), we subsequently determined whether dorsal- and ventral-derived NSCs differed in their capacity to generate GRP cells.

In these experiments, E10.5 spinal cord [at which time point all cells are NSCs (Kalyani et al., 1997)] was microdissected into dorsal and ventral regions. Dissociated cells were plated at clonal density on fibronectin/laminin-coated grid dishes in the presence of 10 ng/ml bFGF and embryonic chick extract (CEE), a condition that prevents differentiation of NSCs (Kalyani et al., 1997). After 3 d in culture, when clones reached a size of 20–50 cells, CEE was removed to allow the clones to differentiate into lineage-restricted precursor cells (Kalyani et al., 1997; Mayer-Pröschel et al., 1997; Rao and Mayer-Pröschel, 1997). After 5 d in the absence of CEE, clones were stained with A2B5 antibody and the number of clones containing A2B5$^+$ cells was determined.

Both dorsal- and ventral-derived NSCs generated A2B5$^+$ cells with a similar efficiency. From a total number of 175 ventral-derived NSC clones, 152 (i.e., 87%) contained A2B5$^+$ cells after 5 d of *in vitro* growth. Similarly, 200 of 213 (84%) dorsal-derived NSC clones cell clones contained A2B5$^+$ cells at this time point. Analysis of the differentiation potential of A2B5$^+$ cells derived from dorsal and ventral NSCs confirmed that these cells expressed the differentiation characteristics of GRP cells (Table 2). These experiments were performed as described previously (Rao and Mayer-Pröschel, 1997). Briefly, clones were stained with A2B5 as live cells and single clones were picked and replated into grid dishes. Single A2B5$^+$ cells were marked and expanded in the presence of bFGF. After clones reached a size of 20–40 cells (5 d), they were switched to 10% FCS to generate astrocytes. After 7 d, clones were stained with A2B5 and anti-

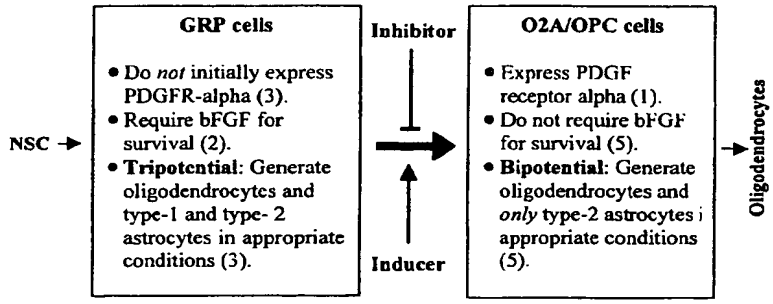

Figure 5. Sequential lineage restriction in the glial lineage of the CNS. A side-by-side comparison of the salient features of two lineage-restricted glial precursors of the CNS is shown. The evidence presented here strongly suggests a progressive and sequential transition from the tripotential GRP cell to the bipotential O2A/OPC. In the developing spinal cord, it currently seems most likely that this transition is controlled in a temporal and spatial pattern and is regulated by cell-extrinsic signaling molecules (Pringle et al., 1992; Rao and Mayer-Pröschel, 1997; Rao et al., 1998).

GFAP antibodies. All astrocyte-containing clones always contained a mixture of type-1 and type-2 astrocytes, regardless of whether they were generated in dorsal- or ventral-derived cultures and whether they were generated in response to FCS (or BMP, data not shown). A smaller number of clones consisted of $A2B5^+$ cells only, and none of these clones contained oligodendrocytes. (Table 2). In contrast, exposure of clones to PDGF plus TH for 10 d was associated with oligodendrocyte generation in all clones. Although differences were not striking, significantly more ventral clones generated oligodendrocytes than did dorsal clones over this time period (35 ± 7% vs 25 ± 2%, respectively; $p < 0.02$).

DISCUSSION

One of the essential challenges that arises with the discovery of any new precursor cell population is to determine how these cells might be integrated into (or might alter) existing views on tissue development. In the present studies on the tripotential GRP cell of the embryonic rat spinal cord, we have found that this recently discovered novel glial precursor cell can generate progeny with the antigenic phenotype and differentiation characteristics of bipotential O2A/OPCs. This process is regulated by cell-exogenous signaling molecules, with growth in the presence of PDGF plus TH being particularly effective in promoting such differentiation. In contrast to previous suggestions that putative oligodendrocyte precursor cells are localized in ventral regions of the E14 spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Timsit et al., 1995; Hall et al., 1996; Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000), GRP cells could be isolated from both the dorsal and ventral cord of E13.5 rats. However, there were differences between dorsal- and ventral-derived GRP cells in their response to conditions that promote or inhibit generation of O2A/OPCs or oligodendrocytes, with ventral-derived GRPs exhibiting a greater propensity to differentiate along the oligodendrocyte lineage.

The demonstration that GRP cells can yield O2A/OPCs integrates these two glial precursor cell populations for the first time and indicates that their relationship is one of sequential lineage restriction rather than being independent precursors that generate oligodendrocytes. In light of our present studies, the simplest model of oligodendrocyte generation that appears to be consistent with all available data would be that production of these cell types requires the initial generation of GRP cells from NSCs followed by the generation of O2A/OPCs from GRP cells (Fig. 5). Our previous studies (Rao and Mayer-Pröschel, 1997; Rao et al., 1998) indicated strongly that GRP cells are a necessary intermediate between NSCs and differentiated glia, and our present studies raise the possibility that O2A/OPCs are a necessary intermediate between GRP cells and oligodendrocytes. Despite the fact that both GRP cells and O2A/OPCs are $A2B5^+$, it seems unlikely that the $O4^+GalC^-$ cells studied in our *in vitro* experiments were derived from a subset of $A2B5^+O4^-$ bipotential O2A/OPCs present in the original GRP cell culture. In our previous characterizations of GRP cells derived from E13.5 spinal cords, we consistently failed to find clones that gave rise exclusively to type-2 astrocytes when exposed to 10% FCS, even when cells were serially recloned three times over a period of several weeks (Rao et al., 1998). Moreover, analysis of hundreds of putative GRP cell clones thus far has failed to reveal clones that generate only type-2 astrocytes when exposed to FCS or BMPs (Mayer-Pröschel, unpublished observations). Thus, it appears that the generation of cells with the characteristics of O2A/OPCs is a differentiation event that requires exposure of GRP cells to appropriate inductive signals, such as PDGF plus TH. Moreover, we could find no $GalC^+O4^-$ oligodendrocytes in any conditions, which would have at least raised the possibility that oligodendrocytes might be generated directly from GRP cells. Such results are consistent with previous observations that passage through an $O4^+GalC^-$ stage of development is required for oligodendrocyte generation from bipotential O2A/OPCs (Gard and Pfeiffer, 1990, 1993; Gard et al., 1995). Our data are also consistent with other studies indicating that $O4^+GalC^-$ cells are bipotential (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999).

It remains formally possible that GRP cells might be able to generate oligodendrocytes without passage through an intermediate O2A/OPC stage, or that NSCs could generate O2A/OPCs without going through a GRP cell stage. Nonetheless, it is important to stress that no data exist to support the possibility that O2A/OPCs are directly generated from NSCs or that oligodendrocytes are directly generated from either NSCs or GRP cells. Thus, the developmental pathway we suggest is at present the only one supported by experimental observations.

It is of particular interest to find that ventral-derived GRPs seem to differ from dorsal cells in such a manner so as to have an increased probability to generate O2A/OPCs and/or oligodendrocytes, even in the presence of BMP. Thus, it may prove necessary not only to study GRP cells but also to focus attention on ventral-derived GRP cells to understand the mechanism of action of those factors that eventually lead to oligodendrocyte generation. It will be of considerable interest to determine whether these differences are intrinsic to ventral- or dorsal-derived GRP cells or are acquired as a consequence of exposure to particular environmental signals. It also will be of interest to determine whether the O2A/OPCs generated from dorsal and ventral GRP cells themselves differ in their responsiveness to

inducers of oligodendrocyte generation, an interpretation that would be consistent with our data (Fig. 4*B*). In addition, our observation that the responsiveness of GRP cells to PDGF plus TH as promoting signals of O2A/OPC and oligodendrocyte generation may decrease with increased GRP cell expansion *in vitro* is reminiscent of our previous findings that O2A/OPCs expanded for continued periods become less responsive to PDGF as a mitogen (Bogler et al., 1990). Although the biological implications of this observation with respect to GRP cell biology require additional investigation, this result does emphasize the importance of expanding precursor cell populations *in vitro* as minimally as possible in studies on the function of exogenous signaling molecules.

It is important to consider the question of whether all previous studies attempting to define the early origin of the oligodendrocyte lineage have in fact been describing early differentiation events affecting GRP cells. It is clear from our previous work that GRP cells can express the PDGFR without losing their tripotential character (Rao et al., 1998). In addition, our ongoing work (Pröschel, Gass, and Mayer-Pröschel et al., unpublished observations) is demonstrating that GRP cells can also be NG-2$^+$ and GD3$^+$, two other antigens that have been used in studies on O2A/OPCs (Mayer-Pröschel, unpublished observations). Moreover, it currently appears that GRP cells are the dominant (if not exclusive) A2B5$^+$ cell population in the spinal cord until as late as E17 (Mayer-Pröschel, unpublished observations). Thus, it is beginning to seem likely that events such as expression of PDGFR in ventral A2B5$^+$ cells may reflect a differentiation process in GRP cells rather than the transition to being an O2A/OPC. Analyzing the early stages of generation of O2A/OPCs from GRP cells, whether *in vitro* or *in vivo*, will require identification of a marker that can be used to antigenically distinguish GRP cells from the A2B5$^+$O4$^-$ stage of O2A/OPCs. As indicated, none of the markers currently available seem to enable this distinction.

The field of developmental neurobiology is in the early stages of determining the relationship between different lineage-restricted precursor cells in the CNS, and our present experiments represent a critical step in determining whether GRP cells may be the ancestors of all glial populations of the spinal cord. Our present observations are consistent in two ways with such a suggestion. First, if this hypothesis were to be correct, then GRP cells should be able to give rise to O2A/OPCs (as we have found). We also would anticipate that GRP cells would be found in both the dorsal and ventral cord, although they may generate different progeny in these two regions. In future studies, it will be important to discover whether precursor cells with the properties of GRP cells also exist in other regions of the CNS. In addition, it will be important to determine whether other progeny of GRP cells include the A2B5$^+$ astrocyte precursor cells present in embryonic (E17) spinal cord and originally described by Fok-Seang and Miller (1992, 1994), the putative astrocyte precursor cells from the embryonic mouse cerebellum described by Seidman et al. (1997), the astrocyte precursor cells described by Mi and Barres (1999), or the pre-O2A progenitor cell described by Grinspan et al. (1990). In addition, it is of importance to determine whether the developmental inter-relationships that seem to exist in the spinal cord also apply to development of the brain. By identifying the relationship between these developmental pathways and the signals responsible for these transitions, we will move closer to a comprehensive understanding of glial development in the CNS.

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